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CONFORMATIONAL CHANGES IN PROTEIN MOLECULES AT INTERFACES

by

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To Azam, Naeem, Asad and My Parents.

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ABSTRACT

The work described in this thesis was undertaken in order to obtain a greater understanding of the processes accompanying the adsorption of proteins. The experiments were made at air/liquid and liquid/liquid interfaces since interfacial energy is directly measurable. The protein chosen was human serum albumin (HSA) since this molecule has a known amino acid sequence. In the case of proteins a very long time, of the order of 16 hours, is required before the interfacial tension reaches its equilibrium value. The diffusion coefficient of HSA indicates that adsorption is complete in a very short time, i.e., about 10^{-3} - 10^{-6} s. The relaxation processes involved in the surface tension changes must therefore be of other origin. These changes are conformational changes in the structure of the molecule and are associated with the process of denaturation.

In order to obtain insight into the unfolding, a detailed study of the surface rheological properties of the adsorbed films was undertaken. A stress relaxation technique was developed which provides information on the surface elastic modulus and the loss modulus over a wide range of frequencies. The results show the presence of two dominant processes with characteristic times τ_1 and τ_2 of the order of minutes and hours respectively.

The variation of τ_1 and τ_2 was determined as a function of pH of the aqueous phase, the bulk concentration and the interfacial concentration, Γ . Γ was measured directly using a radiotracer technique since it became clear that the Gibbs adsorption isotherm gives incorrect values at high bulk concentrations.

The values observed for τ_1 and τ_2 combined with surface and interfacial tension measurements revealed the following properties of films of adsorbed HSA.

1. The degree of unfolding of the molecule is strongly dependent on the pH of the solution.
2. The presence of S-S bridges limits the degree of unfolding.
3. The presence of an apolar phase causes additional unfolding due to the interaction of the hydrophobic groups with the medium.
4. At high concentration the presence of the adsorbed molecules means that newly arrived molecules may not have sufficient space to unfold and so remain in their native state. These molecules return to the bulk phase if the surface is compressed. Hence the molecules exist on the surface in various conformational states.
5. After adsorption is complete the structure of the adsorbed film slowly relaxes to a stress free condition.

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NOMENCLATURE

A	Area occupied by the molecules.
A_c	Specific activity of albumin.
a^*	Activity of the molecules.
a_o	Limiting condensed area per statistical unit of the polymer.
a_1	Arbitrarily chosen area per molecule.
a	Concentration of the monomer.
a_s	Szyszkowski's constant.
β	Damping coefficient.
C	Bulk concentration.
CPS	Counts per second.
D	Diffusion coefficient.
de	Largest diameter of the drop
d_s	Diameter at a horizontal plane distance
δ	Maximum dimension of the molecule.
ΔG^{es}	Energy due to electrostatic interaction.
ΔG^{lw}	Sum of long range forces.
ΔG^{tot}	Total free energy of the interface.
ΔG^{sr}	Sum of short range forces.
E_b	Concentration of non surface active radionucleides present.
E_s	Concentration of surface active radionucleides present.
ϵ	Dilatational modulus.
ϵ'	Storage modulus.
ϵ''	Loss modulus.
ϵ_d	Dilatational elasticity.
ϵ_o	Limiting value of the modulus.
ϵ_s	Shear elastticity.

f	Bound fraction.
g	Acceleration due to gravity
γ	Surface tension.
γ^d	Dispersion forces of surface tension.
γ_o	Surface free energy
γ^p	Polar forces of surface tension.
Γ	Surface excess concentration.
k	Wave number.
κ	The correction factor
κ_{av}	Stationary volume.
L	Distance between the barrier and end of the trough.
λ	Wavelength.
m_s	Concentration of surface active radionucleides added.
m_b	Concentration of non surface active radionucleides added.
N	Number of molecules.
N_1	Degree of polymerization.
N^o	Number of a solvent molecules.
η_i	Surface concentration of component i.
η_d	Dilatational viscosity.
η_s	Shear viscocity.
ν	Frequency.
ν_1	Number of amino acids in direct contact with the surface.
θ_s	Surface coverage.
θ_*	Volume fraction of the polymers.
θ_c	Contact angle.
P	Pressure.
p	Perimeter of the plate.

π	Surface pressure.
R	Gas constant.
r	Number of segments in a polymer chain.
ρ	Density.
S	Entropy of the system.
S_g	Radius of gyration.
s	Shape of the drop.
T	Absolute temperature
t	Thickness of the adsorbed polymer layer.
th	Hydrodynamic thickness of the adsorbed polymer layer.
t_{rms}	Root mean square thickness of an adsorbed polymer layer.
t_o	Time.
t_1	Number of amino acid residue/protein molecules.
τ	Characteristic time.
θ	Phase angle.
U	Total energy of a system.
u	Chemical potential.
ζ_B	Amplitude of the oscillation of the barrier.
V	Volume
V_e	Elution volume.
V_o	Void volume.
V_t	Total volume.
W	Wave propagation number.
W_o	Work done.
ω	Angular frequency.
χ	Segment-solvent interaction parameter.
x	Number of statistical units per polymer molecule.
Z'	Lattice co-ordination number.

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CHAPTER ONE

THE ROLE OF ADSORBED PROTEINS IN DETERMINING BIOCOMPATABILITY

1.1 INTRODUCTION

During the last few years a large number of polymers have been used as biomaterials for various purposes such as tissue implants and in replacement of parts of the cardio vascular system.

The biocompatibility of these polymers has been widely investigated by Baier *et al.* (1968, 1969) and only those polymers which provoked a minimal response when exposed to the physiological condition are considered to be biocompatible.

When a polymer surface comes into contact with blood it causes:-

1. protein adsorption;
2. platelet adhesion and aggregation;
3. activation of the intrinsic coagulation.

Complete knowledge of the events that occur during blood-polymer contact is not available but it has been shown that the above three processes play a vital role in the characterization of a biocompatible material. Kim (1976) has suggested that adhesion and aggregation of platelets on an implant do not occur directly on the polymer surface but occur only on a surface on which a layer of plasma proteins has adsorbed. Therefore the adsorption of plasma protein is regarded as an important precursor of the platelet adhesion and aggregation. The nature of blood-polymer interaction is exceedingly complex and is far from being completely understood but good progress in the development of non-thrombogenic material has only been achieved by consideration of the interfacial processes that precede and initiate platelet

adhesion. Different polymer surfaces are selective in the proteins that they absorb. From the detailed analysis of the extent of platelet adhesion and the nature of the adsorbed protein layer *Kern* suggests that platelets adhere to adsorbed fibrinogen and gamma globulin molecules. These adsorbed proteins form a complex structure between their incomplete heterosaccharide moieties and the glycosyltransferases present on the platelet membrane. As albumin does not have any such moieties it does not promote platelet adhesion. Also from studies done in vitro by Lee *et al* (1974) it has been shown that thrombogenic polymers tend to adsorb fibrinogen and gamma globulin from mixed solutions with albumin, but non-thrombogenic polymers such as polyether urethanes adsorb albumin preferentially.

The events leading to thrombogenesis takes place rapidly perhaps in a matter of minutes but the adsorption of plasma protein takes several hours to reach equilibrium. The extent of changes in the initially bound adsorbed layer reflects the chemistry of the polymer. If the polymer is inert then it will have a minimal response to its surroundings.

A study on segmented polyurethanes by Russell *et al*. (1981) showed that increasing hydrophilicity resulted in decreased reactivity with adsorbed protein and hence with platelets, provided no formation of hydrogen bonds or other polar interactions occurred, in the structure of the polymer. It is also shown that an increase in the side chain length of acrylates and methacrylates increases their capacity for hydrophobic interactions with adsorbed plasma proteins which is responsible for the increased platelet reactivity to these polymers. This shows that protein modifies the surface of the

foreign material and hence plays a key role in determining the biocompatibility of a material.

1.2 EFFECTS OF PROTEIN ADSORPTION ON THE ELECTRICAL CHARGES

The electrical charge on the surface of the polymer was originally thought to be of importance because of the naturally occurring negative potential on the vascular surface. Hence efforts were made to correlate the zeta potential of different polymers in contact with Ringer's solution with the thrombogenicity of the surface but Leininger *et al.* (1964, 1968) showed that such a correlation could not be established as the initially negative zeta potential of the polymer shifted rapidly when exposed to blood, due to the adsorption of blood proteins on the polymer surface.

Horan *et al.* (1950) and Bull (1962) also studied zeta potential and electrophoretic behaviour and demonstrated that the charge on a surface does get affected by adsorption of proteins. For example a polymer can find its surface charge not only neutralized but reversed when it adsorbs an excess of albumin, so that when it appears to be coated with a layer of protein it has the same electrophoretic mobility as the bulk protein.

Therefore adsorption of proteins modifies the interaction of the artificial surface with the cellular elements. Hence certain thrombogenic polymers can be made compatible if a layer of serum albumin is attached to it before being exposed to blood. This method of passivity of otherwise incompatible surfaces has shown some promise in the synthesis of prosthetic materials.

As it is generally accepted that the vascular wall lining is an anionic mucopolysaccharide (Lyman 1966, 1968; Sawyer *et al.* 1964),

therefore clot initiation on foreign surface can also be attributed to ionic charges provided by the surface. Lyman (1968) has reported that anionic surfaces retard or eliminate clotting.

1.3 EFFECTS OF PROTEIN ADSORPTION ON WETTABILITY

Wettability, which is another surface characteristic, was also investigated. Lampert (1930) determined that the coagulation time of blood on a variety of surfaces - glass, metal, paraffin etc., is inversely proportional to the wettability of the surface by water or other liquids. However, On glass surfaces which are quite wettable, coagulation occurs much faster than on a surface coated with the relatively non-wettable paraffin. Moreover, Rose & Broida (1954) from their findings of whole blood clotting time on a variety of surfaces concluded that Lampert's simple concept does not hold. Ross *et al.* (1961) also showed the inadequacy of the concept of Lampert relating clotting to the wettability of the surface. Since several substances can be adsorbed on the surface therefore it is not possible to simply correlate wettability with clotting time.

Adsorbed proteins also tend to affect the wettability of various interfaces. Some proteins such as thrombin and fibrinogen when adsorbed decrease the wettability of high energy surfaces and increase the wettability of low energy polymeric materials (Vroman, 1962; Vroman *et al.* 1971).

Ghosh *et al.* (1964) have shown that if adsorbed protein films cannot be easily removed then it indicates that hydrogen as well as hydrophobic bonds are involved. However, if only hydrophobic bonds are involved, for example, when adsorption of protein occurs on paraffin, then they can be easily removed.

Andrade (1973) has related adsorption of proteins to interfacial free energy and suggests that adsorption of plasma proteins occurs when interfacial free energy between the implant surface and the blood is high. In the absence of high interfacial free energy such adsorption would not occur and the materials exhibiting these properties would be non-thrombogenic. Andrades hypothesis about minimum interfacial free energy led to the development of hydrogels for blood compatible prosthesis. Although successfully used for various medical applications such as drug delivery systems and for soft tissue implants they have not provided a complete answer to the development of ideal long-term vascular prostheses. This is also at least partly connected with their other properties such as mechanical strength. Hydrogels are known to be mechanically weak and so not suitable for implants which take a lot of strain. Glass which interfaces well with saline solution and is wetted by blood is found to be thrombogenic and this has been used by some researchers to cast doubt on the concept of interfacial free energy being responsible for thrombogenicity. However, even though the glass is wetted by blood, the interfacial tension between glass and blood need not be low and probably lies between the surface energy of glass/air ($\sim 500 \text{ mJm}^{-2}$) and water/air (72.8 mJm^{-2}).

1.4. THE EFFECTS OF DESORBABILITY

From studying desorbability of proteins from solid surfaces into pure buffer it has generally been concluded that desorption from hydrophobic surfaces does not occur (Brash & Lyman, 1969; Morissey *et al.*, 1977; MacRitchie, 1972). Desorbability from hydrophilic

surfaces has been shown to depend on conditions of pH relative to the isoelectric point. Desorption of albumin from silica has been shown (Macritchie, 1972) to occur at pH values far removed from isoelectric point. Also Coleman *et al.* (1976) have shown that preformed albumin layers on glass and polystyrene are considerably depleted after 24 hr in contact with plasma. Whether the original albumin is replaced by albumin or other protein is not indicated. However the existence of a turnover process is interesting with respect to blood surface interactions. Therefore Brash *et al.* (1978) studied the effect of flow and solution concentrations on the turnover of albumin adsorbed on polyethylene. They studied surface concentration as a function of time of contact. The basic method used to determine the turnover was by allowing adsorption to attain steady state using one form of labelled protein I¹²⁵ and then changing to the other form without changing the concentration. The extent of turnover was determined by the decrease in the second form on the surface. The results from the loss and gain curves indicated that there is a fraction of the surface layer which is exchangeable and the remainder is not. The exchangeable fraction increases with concentration. This was attributed to the large number of binding possibilities between a heterogeneous polyethylene surface and a protein molecule. These would range from various van der Waals attraction to hydrogen and covalent bonding. However their results stressed the dynamic character of protein adsorption and suggested that surface concentration could change in composition with time thus indicating that thrombus formation is influenced by the slight changes in plasma protein composition of the adsorbed layer.

The work described later in this thesis suggests that the reason for this is that the molecules which adsorb initially are irreversibly denatured while those arriving later when the surface is nearly saturated adsorb in a conformation which is close to the native state. It is these molecules which exchange easily. The work leading to this conclusion is described in Chapters Four and Five.

Therefore to understand biocompatibility, it is not only essential to determine the relationship between the nature of the adsorbed protein layer and the biomaterial but also the nature of the bound protein layer, in particular whether the protein layer is simple or multilayered, and whether adsorption is reversible or not. Also the conformational state of the adsorbed protein molecule is important as it will ultimately determine the extent of the native like appearance of the protein covered implant.

1.5 CHARACTERIZATION OF THE ADSORBED MONOLAYER

It has been suggested by Lyman *et al.* (1971) and Brash *et al.* (1969) that the adsorbed monolayer of individual proteins on hydrophobic surfaces approximates in thickness to one of the linear dimensions of the intact globular molecule, indicating that protein adsorbs with minimal conformational change. William & Bagnall (1981) attempted to explain this by considering adsorption to be multilayered so that an unfolded monolayer backed by an intact globular monolayer might be experimentally indistinguishable from a simple intact globular monolayer.

From competitive adsorption experiments of the major plasma proteins it has been shown that fibrinogens dominate the adsorption phen-

omenon on hydrophobic polymers (Lee *et al.* 1974; Vroman *et al.* 1967). The concentration of fibrinogen, albumin and γ -globulin in plasma are in the ratio of 4:46:12 and their molecular weights are 340,000:69,000:150,000 respectively. As initial adsorption is controlled by the diffusion process, it would be unfavourable for the fibrinogen molecule to be adsorbed faster because of its low concentration and high molecular weight. William & Bagnall also explained these results by assuming the formation of a protein double layer instead of a single intact globular layer. This would mean that an unfolded first layer and an outer layer of globular protein is formed. The ability of fibrinogen to unfold on adsorption would then promote contact between individual molecules, a process that would not occur in an intact globular molecule. The rate and degree of unfolding of an adsorbed fibrinogen molecule are also dependent on the surface on which they are adsorbed. Unfolding may be hindered due to the presence of other adsorbed molecules such as albumin and γ -globulin thus desorbing them and giving a fibrinogen dominated surface film. It is observations of this kind which stressed the importance of understanding the whole process of adsorption from the initial fully unfolded state to the equilibrium configuration.

1.6 CHARACTERISTICS OF A BIOCOMPATIBLE POLYMER

The list of polymers tried or used in medical applications is extremely long and they are diverse in nature, therefore it would be unlikely that the mechanism of blood compatibility would be the same for each type. Baier *et al.* (1970) carried out an important series of experiments in which the surface characteristics of several implan-

table materials was studied. Critical surface tension, spreading pressure and infrared internal reflection spectra were obtained before and after implantation. He suggested that at least three different mechanisms can be correlated with thromboresistance of various materials.

1. Intrinsically low energy surfaces, such as obtained with tallow polished stellite which apparently resist thrombus formation because of an erosion resistant, biocompatible coating of closely packed methyl groups.
2. Intrinsically high energy surfaces, such as diamond which rapidly adsorb thin protein films from blood and convert their surfaces to a thromboresistant low energy character.
3. Materials with desorbable surface active agents (surfactants, heparin albumin etc.) so that they will slowly and continuously leach into adjacent liquid phases and thus shed thrombus precursors as well.

However in all three instances, a proteinaceous layer usually deposits on implant materials rapidly and spontaneously and it is this layer which determines subsequent substrate reactivity to blood.

1.7 AIMS OF THE PRESENT WORK

The view that biocompatibility of polymeric material is greatly influenced by protein adsorption formed the basis of the work described in this thesis. Adsorption of proteins is a slow process and it takes several hours before it reaches equilibrium. However, thrombogenesis in implants when exposed to blood occurs within seconds or

minutes suggesting that it occurs under non-equilibrium conditions. The experiments of Baier *et al.* highlighted the importance of interfacial parameters and suggested that ideal biocompatible materials should present a low interfacial tension to blood. If the interfacial tension is high either because the implant has very high or very low tension compared with blood, adsorption of protein will occur at the solid/liquid interface which reduces the interfacial tension and hence renders the interface more compatible.

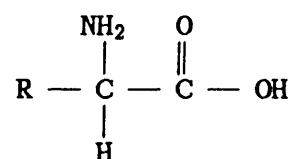
The conclusions of various workers are often not consistent and in addition few have investigated the changes which occur when pH is varied. Since proteins are polyelectrolytes it is to be expected that some conformational changes accompany changes in pH of the medium. The effects of this parameter were therefore included in the present work. The difficulties associated with making measurements at solid/liquid interfaces which are evident from the experiments of Baier *et al.* suggested that more detailed study might be possible by working at liquid/liquid interfaces. Changes in the surface and interface tensions of HSA solutions, as various parameters were varied, were studied using a number of techniques which are discussed in Chapter Three. These observations showed the importance of independent surface concentration measurements. Surface rheological techniques originally devised to study simple surfactant solutions at air/water surfaces were modified to be used at liquid/liquid interfaces and applied to the study of this protein. The molecule undergoes many complex changes which are reflected in these measurements. They are described in Chapters Five and Six.

CHAPTER TWO

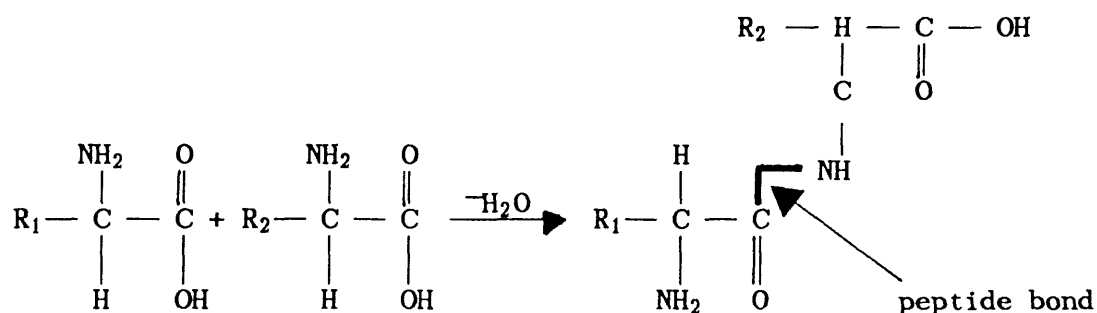
THE STRUCTURE AND CHARACTERISTICS OF ADSORBED PROTEIN FILMS

2.1 INTRODUCTION

Proteins are one of the major class of compounds found in all living matter. They are natural polymers of high molecular weight having chemically complex subunits, the amino acids. Amino acids are molecules with an average molecular weight of about 120, each of which has an organic acid group and a basic amino group -NH_2 . The acidic and basic groups are attached to the same carbon leading to the formula



Two amino acid molecules react to eliminate a molecule of water thereby forming a peptide bond. Schematically this is represented as:



The peptide bond formed is very stable and the resultant molecule is called a dimer or dipeptide. When more amino acid molecules are

attached to the chain they are called polypeptides. Naturally occurring chains which include more than 50 subunits are called proteins. Each type of protein has in its native state a characteristic three dimensional shape, referred to as its conformation. Proteins can be placed in two major classes, depending on their conformation, viz. a) fibrous protein and b) globular protein.

2.1.1. Fibrous proteins

These are insoluble in water or in dilute salt solutions, and consist of polypeptide chains arranged in a parallel fashion along a single axis, to yield long fibres or sheets. They are generally associated with structural functions.

2.1.2 Globular proteins

These consist of polypeptide chains that are tightly folded into compact spherical or globular shapes. Generally the molecule has polar groups on the surface whereas the interior consists of non-polar hydrophobic groups. Globular proteins normally have metabolic functions for example as enzymes or as transport proteins.

2.2 PLASMA PROTEINS

There are three major plasma proteins: albumin, globulin and fibrinogen. From x-ray diffraction studies albumins are classified as globular proteins. Fibrinogen as fibrous and globulins, unlike other proteins, are found to exist in either a fibrous or globular state (Putnam 1975).

The properties of a protein are mainly determined by the side chains, and thus by the nature and sequence of the amino acid residues

which gives the primary structure. With the exception of proline and hydroxiprolin, all amino acids have the L-configuration. The α -carbon is asymmetric - this is reflected in the steric structure of the protein molecule which constitutes the secondary structure.

It has been demonstrated for many proteins that an α -helical configuration is favoured, and a number of proteins found in nature (myoglobin, albumin, myosin, collagen, tropomyosin, α -lactalbumin, α -casein) appear to confirm, either wholly or in part, to such a coiled-spring structure. The shape of the helical pattern (Fig. 2.1) is formed by the hydrogen bonds. Here hydrogen, bound covalently to nitrogen or oxygen, tends to form weak bonds with other oxygen or nitrogen atoms. Other atoms are also capable of participating in hydrogen bonding, but the NH ---O< type is the most important in proteins. In the α -helix configuration, each amino acid is bound to each fourth one by a hydrogen bond (Fig. 2.1). The helix conformation preeminently provides for maximum hydrogen bonding which maintains the coiled spring pattern, which gives a minimum distortion of bond lengths and bond angles in the peptide chain.

Some proteins contain no α -helical segments. Instead, they have many residues that are in an extended, zig-zag conformation of the polypeptide chain. This has been designated the β -conformation. Parallel chains in the β -conformation form structures, called pleated sheets, which are cross-linked by interchain hydrogen bonds (Fig. 2.2).

More decisive for the properties of a protein are a number of other bonds arising from the side chains, e.g. covalent disulphide bridges between two cystein residues, hydrophobic bonds between residues such as glycine, leucine, isoleucine, valine etc. and salt bridges between charged groups of opposite sign. These bonds determine

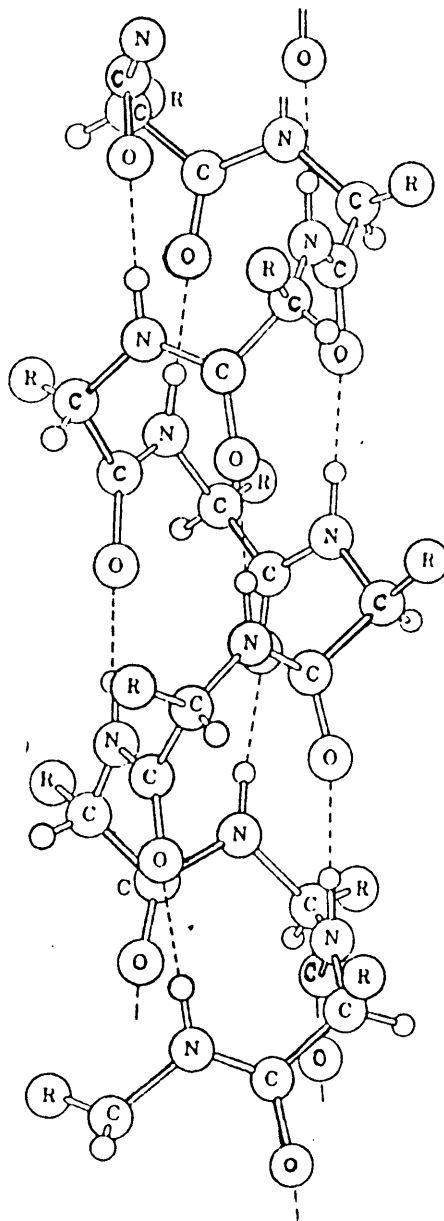
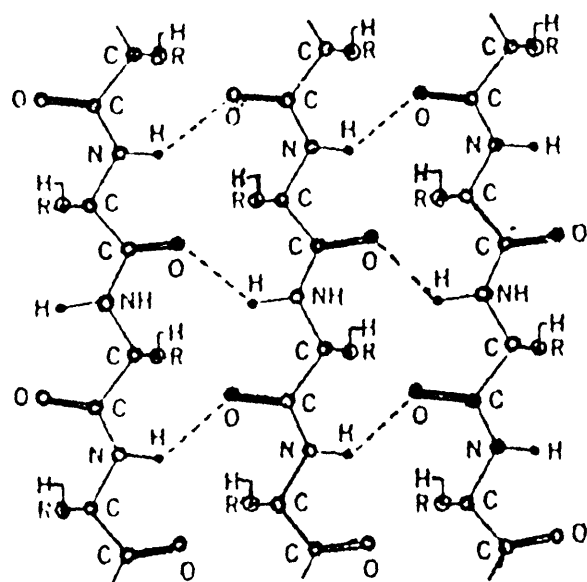
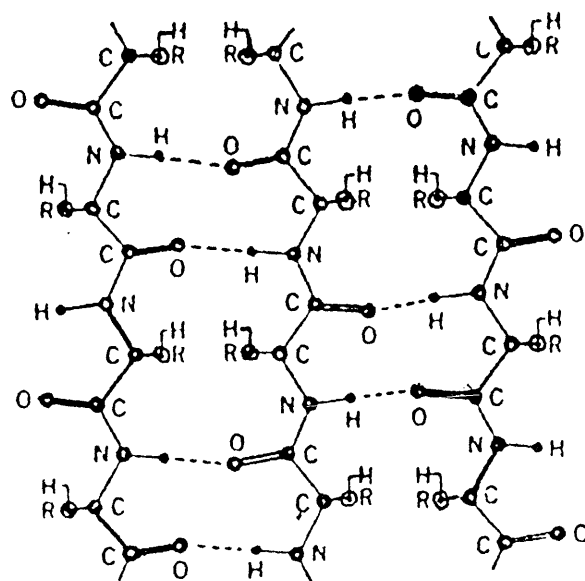


Fig. 2.1 The α -helix configuration (Steiner 1968).



(a)



(b)

Fig. 2.2 The β -configuration (a) parallel chains (b) antiparallel chains (Steiner, 1968).

how the coiled backbone is bent and folded into the ultimate protein configuration (tertiary structure). The plasma protein investigated here is albumin.

2.2.1 Albumin

Compared with other plasma proteins, serum albumin is found in abundance and in relatively pure form. It constitutes $60 \pm 4\%$ of the total serum protein and is often regarded as a more or less homogeneous protein (Eugene). Albumin does show some micro heterogeneity which has been detected by electrophoresis around pH4. It also contains about 2-3% of other polymeric forms. The protein content of blood may increase under some pathological conditions.

Recently as many as 20 albumin varieties have been detected (Allen, 1977). However the physico chemical properties are similar in all species from which it has been isolated (Putnam, 1975) and therefore it has been a model protein for physical chemists who have studied its bulk behaviour by every available technique making it one of the most widely characterised proteins.

2.2.2 Physiological roles

Serum albumin has numerous physiological roles. It is mainly responsible for maintaining the osmotic pressure of the blood and for transportation of various substances. Its normal level in blood is 42 ± 3.5 gm litre⁻¹. In fact its normal level in blood is a measure of health. Only about 40% of the total albumin is normally found in circulation, the remainder is found in the extra vascular regions. About 5% of it is found leaving the blood every hour and is then main

ly returned by the thoracic duct during the next 24-28 hrs. Distribution of albumin in the body is shown in Table 2.1.

Table 2.1
Distribution of Albumin in the Body

Organ	Amount (gm/70-kg man)	Concentration (gm/kg organ)
Vascular Blood	140	24
Extravascular		
Muscle	50	2.3
Skin	40	7.7
Liver	2	1.4
Gut	8	5
Other tissues	110	(3)
Total extravascular	210	
Total body	350	

Estimated from isotopic and immunochemical data of Rothschild *et al.* (1955), Humphrey *et al.* (1957), Dewey (1959), and Katz *et al.* (1970).

2.2.3 Composition of Albumin

Serum albumin consists of a single polypeptide chain of 584 amino acid residues. Its structure was mainly proposed by Brown and his co-workers (1974; 1975), although there are other independent contributions, Shearer *et al.* (1967), King & Spencer (1972), Meloun *et al.* (1972) Kusnir *et al.* (1973a), Kusnir *et al.* (1973b), to the elucidation of the sequence of amino acids residues in the peptide chain. Its molecular weight as determined from the various composition and sequence data ranges from 66,460-69,000 daltons. The structure of albumin reveals that high levels of glutamic acid, aspartic acid and

lysine cause the molecule to be polar. Compared to other proteins it has a high cystine content but levels of methionine, tryptophan, isoleucine and glycine are low (Peters, 1970). Pure albumin is colourless but concentrated solutions of human albumin prepared from homogenized blood may be yellow due to traces of hematin that are difficult to remove (Rosenfeld *et al.* 1950). Albumin is known to have one sulphhydryl group and has 17 inter chain disulphide bonds but is devoid of carbohydrates (Eylar, 1965). It exists in multiple loop form which is responsible for its flexibility or conformational adaptability. Amino acid sequence of bovine and human serum albumin is shown in Figs. 2.3 and 2.4.

2.2.4 Physiochemical properties

The hydrodynamic properties of albumin have been determined in detail (Hughes, 1954; Longworth 1949). Sedimentation, diffusion, viscosity, light scattering and several other methods have been used in these studies. The data obtained indicate that the molecule is asymmetric, an ellipsoid of rotation with a large axis of 14 nm, and a short axis of 3.8 nm.

Albumin is one of the most soluble plasma proteins, solutions containing up to 30 per cent of albumin can be prepared. The high solubility of albumin is probably connected with the large number of ionizable groups present in the molecule. At the isoelectric pH there are more than 200 positive and negative charges distributed over a molecule which gives it a hydrophilic character. Since the number of carboxyl groups is somewhat larger than the basic group, the isoelectric point is at the lower pH of 4.7 (Hughes, 1954).

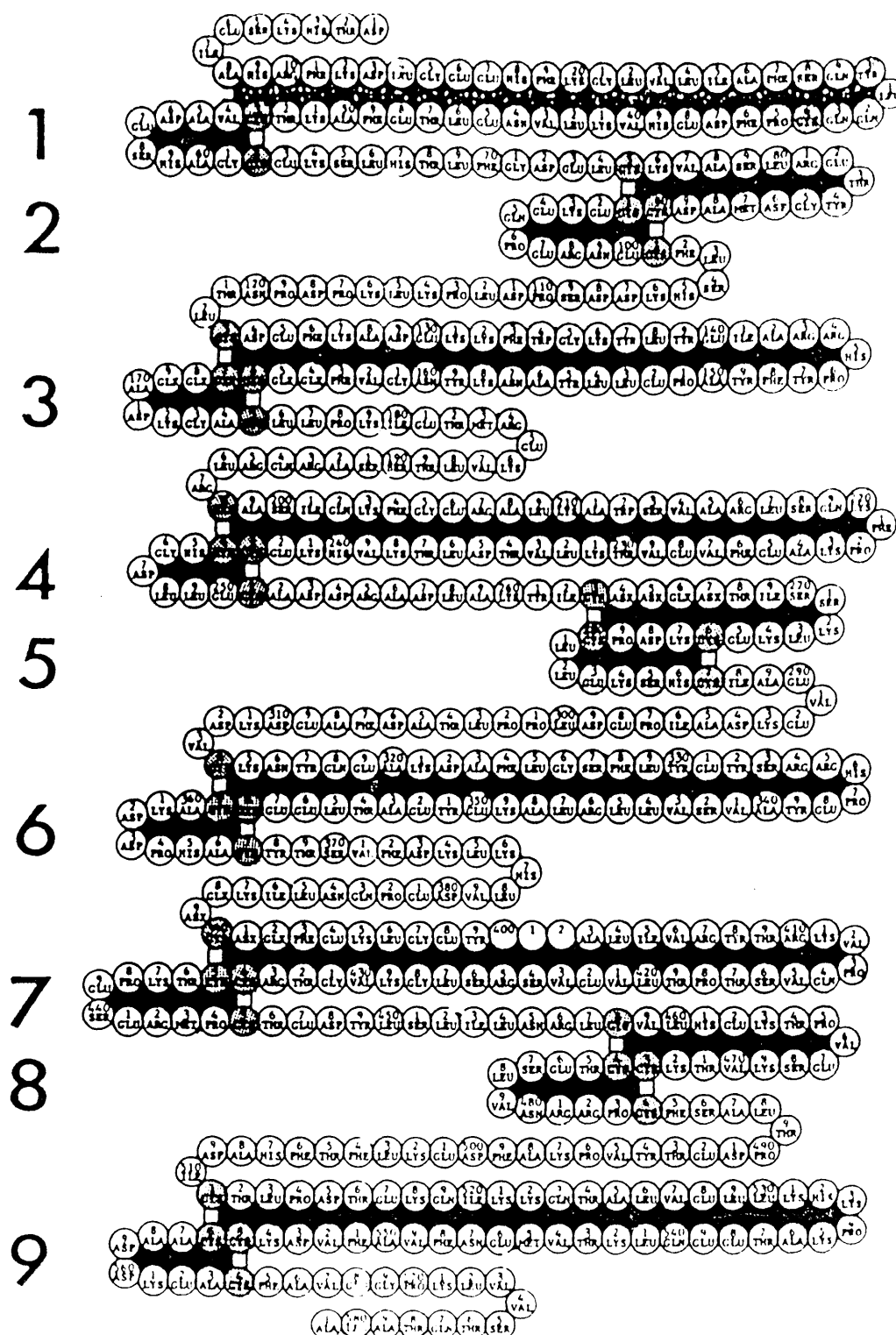
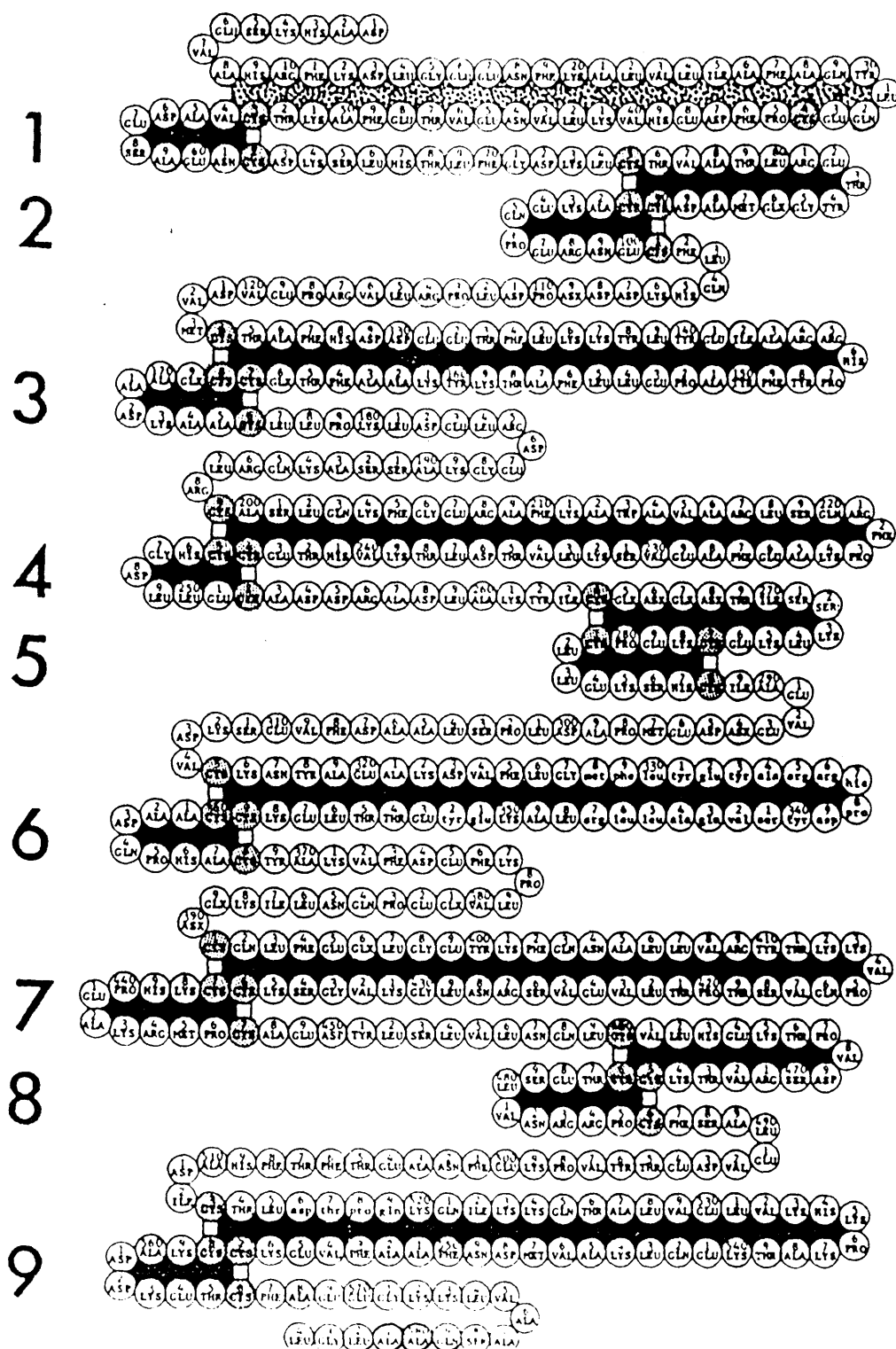


Fig. 2.3 Amino acid sequence of bovine serum albumin, displayed in a model showing the proposed linking of cystines to form multiple double loops. The sequence is complete except for residues 400-402, the residues Phe-Gln-Asn, respectively, were assigned to these sites by analogy with the sequence of human albumin. From Brown (1975). Reprinted from *Fed. Proc.* 34, 591 (abstr. 2105), 1975.



At this pH the molecule is observed to be in a compact state possibly due to the strong associative forces between some of the loops. It is likely that association of the loops forms globular parts or domains and a model showing a linear arrangement of such chains has been proposed by Anderson *et al.* (1969).

The size and shape of the albumin molecule is somewhat dependent on pH. When change in pH occurs from 4.5-3.5 there is a reversible expansion of the molecule. It probably begins as a separation of domains. Primary separations into two (Slayter, 1965), three (Bloomfield, 1966) or four (Weber *et al.* 1964) domains have been suggested. The separations become longer and more asymmetric without a change in molecular weight (Champagne, 1957). The helical content decreases and the molecule unfolds so that the interior parts become accessible. With the exposure of hydrophobic regions the molecule becomes less soluble in aqueous solutions and more soluble in ethanol (Levine, 1954). The full expansion of the molecule occurs in strong acid or 8M urea (Kauzman *et al.* 1953).

A conformational change also takes place in the alkaline pH region. Here alteration in size and shape begins to occur at pH9 (Stroupe *et al.* 1973). As the pH rises to 11.4 the molecule unfolds to expose 11 of the total 18 or 19 tyrosines and is similar to the behaviour in acid (Steinhardt *et al.* 1973).

The accessibility of the 17 S-S bond to reduction or oxidation varies with pH. (Disulphide bonds do not react between pH5 and 7 not even in reagents as strong as 0.2M thioglycolate (Alexander *et al.* 1960). In either acid or alkaline conditions the S-S bonds become progressively more reactive causing gradual unfolding. For complete

reduction, denaturing agents are required (Hunter *et al.* 1959). The reduced and carboxy methylated molecule behaves hydrodynamically as a thread about 2140Å in length with complete random structure but without change in molecular weight (Tanford *et al.* 1967).

The sulphydryl group is not readily oxidized between pH5 and 7. However it reacts with L-cystein and L-cystine to form mixed disulfides under alkaline conditions. The rate of reaction is maximal at pH8.1 (Edward *et al.* 1969).

The titration curves of albumin obtained by Tanford (1962) in the pH range 2-12 are found to be a composite of the ionizable groups of the constituent amino acid residues. The curves are steep in the acid region but flatten near neutrality where only imidazole groups are either giving or losing protons. Thus, albumin is a strong buffer at pH4 or 10 but a weak one at pH7. The slope of the curve in the pH7 region is about 7.3 H^+ per pH unit, therefore about 0.24meq of NaOH are required to adjust a solution containing 1 gm of albumin from pH5.2 to 7.4. In normal plasma, the contribution of albumin to the buffer value is 4.8 meq per litre per pH unit change.

The isoionic point of albumin is near pH5.2. It is also the pH of maximum total charge about 101 +ve and 101 -ve charges. Albumin associates with a large number of compounds having greater affinity for anions as compared to cations. Substances such as fatty acids and bilirubin are highly insoluble at neutral pH and their presence in plasma is only due to their binding affinity to albumin. Albumin also binds to thyroxine and numerous steroid hormones. Many drugs and organic dyes also bind to albumin (Putnam, 1975). The study of drug binding is gaining importance as it provides a more scientific basis for the estimation of drug dosage.

Albumins are good antigens and though the number of antigenic sites in an albumin molecule is not certain at least 7 are known to exist from the precipitation data of Kabat *et al.* (1937). The conformational changes tend to expose or create additional sites.

2.2.5 Synthesis of Albumin

Albumin is synthesized almost exclusively by the liver and only in trace amounts by the thyroid glands (Miller *et al.* 1951; Otten *et al.* 1971). Although the liver is the source of albumin there is less than 2 gms of albumin stored within it. Even this is mostly in the liver lymph as the content in the "true" cytosol has been estimated at 50 $\mu\text{g/gm}$ liver or less (Gordon *et al.* 1961; Peters, 1962a). Approximately 17 gm/day of albumin is synthesized. The daily degradation rate is about 14 gms which is about 10% of the total protein replaced in the body.

Albumin is considered to be an old protein in terms of phylogenetic history and is known to occur as far back in the evolutionary scale as the teleost fishes. Its unit evolutionary period has been estimated as 2-3 million years (Wallace *et al.* 1972). In the few instances in which the albumin of earlier forms have been studied their properties were noted to be quite similar to those of mammalian albumin (Masat *et al.* 1968).

2.3 GENERAL ASPECTS OF ADSORPTION OF PROTEINS

Adsorption of polymers and especially of proteins at various interfaces is of major importance in biology and technology. Unfortunately investigation of the structure and functions of proteins and

polypeptides is much more difficult at interfaces than in the bulk of the solution. Therefore few studies have been conducted at interfaces. Of the three interfaces namely the air/liquid, liquid/liquid and liquid/solid that have been studied the air/liquid interface has been most widely investigated.

2.3.1 Adsorption at air/liquid interfaces

Extensive early work on protein films was carried out by Langmuir (1938, 1939), Harkins (1940, 1944) and Gorter (1941). Most of their studies were conducted on enzymes at the air/water interface for very dilute surface concentration. The films formed corresponded to 'gaseous' films so that the relationship between pressure (π) and area (A) could be described by a two-dimensional gas equation. Minimal interaction was found between the adsorbed molecules. When compressed the film pressure increased very rapidly as the film became closely packed and the surface concentration (Γ) corresponded to about 1 mgm^{-2} . The thickness was found to be approximately equivalent to that of a single polypeptide chain. Hence the adsorbed protein molecules in the gaseous films are unfolded with their polypeptide chains oriented approximately flat in the plane of the surface with the hydrophilic side chains towards the aqueous phase and the hydrophobic side groups away from it. Such configurations, being the equilibrium configurations, minimize the free energy of the system. Under these conditions, where molecules have minimal interaction with each other, they tend to lose their enzymic activity and it is not regained upon compression of the film. However, loss of activity is not observed when molecules adsorb at an interface already occupied by a close packed film of protein molecules (James *et al.* 1966).

Upon compression of dilute films, the film pressure increases greatly as the surface concentration is increased above 1 mgm^{-2} ($\pi > 1 \text{ mNm}^{-1}$). Further compression leads to the collapse of the film (at $\pi > 15\text{--}20 \text{ mNm}^{-1}$) and eventually to the formation of a microscopic thread. The protein films do not exhibit phase transitions (e.g. liquid expanded, liquid condensed, solid) comparable to those described for lipid films. Instead, the close packed protein films have coherence and elastic properties most characteristic of a gel. In general, there is little hysteresis observed when π vs A curves are plotted for compression and expansion of close packed films. Bull (1947) has measured the surface aging of a compressed film. He attributed the changes in π to either slow dehydration of the film or solubilization of low molecular weight fragments in the support solution.

According to Augenstein *et al.* (1958), when films of higher concentration are formed their film pressure measured for a given surface concentrations are far less than that determined when a dilute film is compressed to give the same concentration. These results are an indication that irreversible conformational changes occur in dilute films. From their experiments with trypsin they found that if 2 mg of trypsin is spread on 1 m^2 of 15% $(\text{NH}_4)_2\text{SO}_4$ the film pressure measured after 5 min is 4.5 dynes/cm. By contrast if 0.6 mg is spread on 1 m^2 of this solution and within 1 min the film is compressed to 0.3 m^2 , a π of 12 dynes/cm is observed after 5 min which clearly shows that the dilute films when close packed are less compressible than the concentrated films in a comparable concentration range (see Fig. 2.5). Augenstein *et al.* (1957) also observed surface aging by concentrated

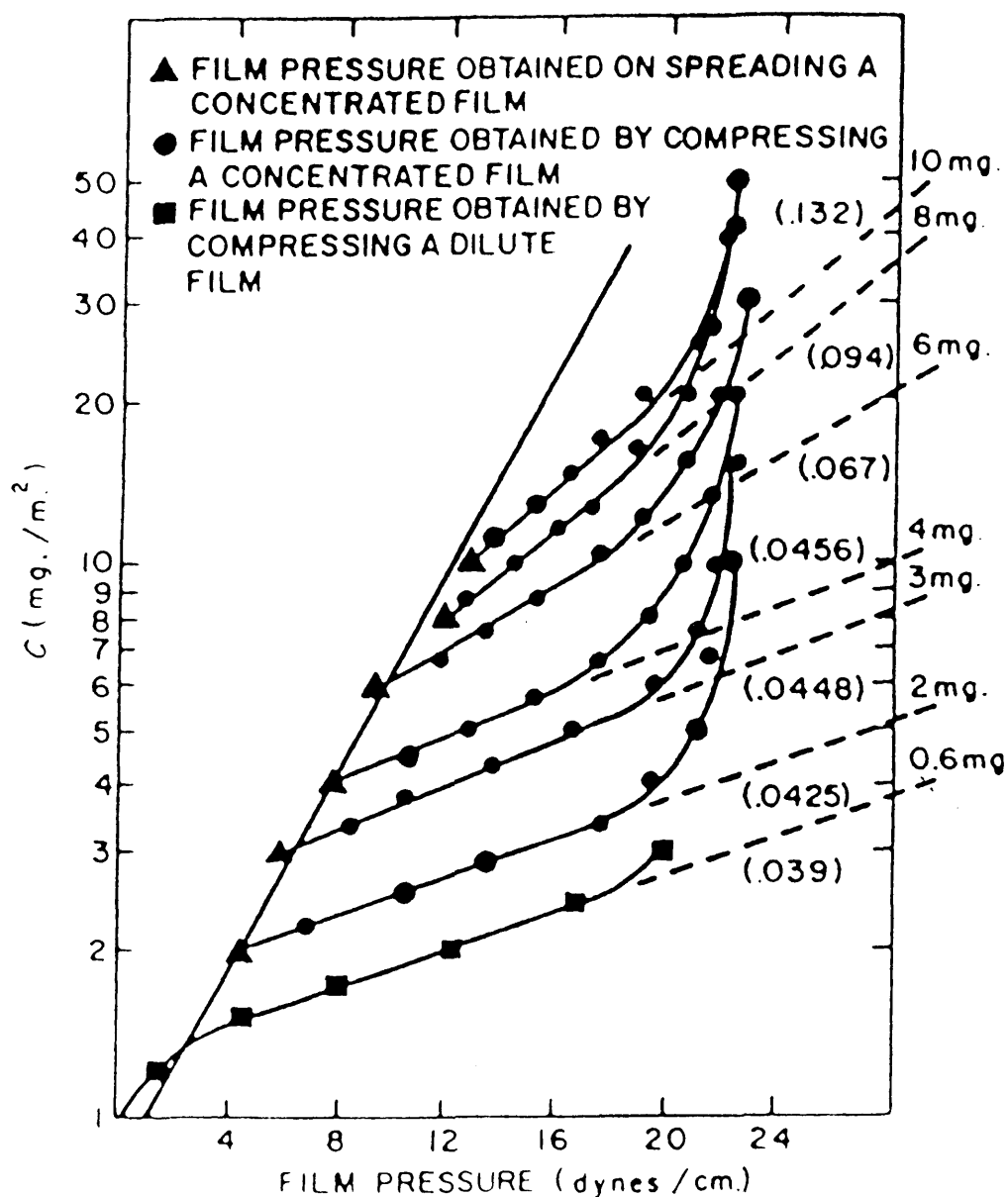


Fig. 2.5 The film pressures produced by spreading and compressing trypsin films on pH4.5 ammonium sulfate (15%) support solution. There were 10 mins. between spreading and the first compression and then 5 mins. between successive compressions and measurements of π . Each curve indicates the results for 6-8 compressions of one film. The number of parentheses are values of compressibility for each film, the numbers at the right show how much trypsin was spread over 1 m² of surface initially (Augenstein *et al.* 1958).

films and measured changes in the values of τ for 3 days. They also recorded large hysteresis when concentrated films are expanded and then compressed. However if they are expanded to surface concentrations less than 1 mgm^{-2} then complete unfolding of the molecules occurs to give dilute films and the τ -A curves thus obtained upon compression were found to be typical of the gaseous type of films, indicating that conformational changes must occur at the interface.

2.3.2 Adsorption at solid/liquid interfaces

The literature pertaining to the study of protein adsorption at solid/liquid interfaces presents much evidence for conformational changes accompanying adsorption but no clear picture for the adsorption mechanism has yet been developed.

Ulman *et al.* (1952) gave some basic concepts of polymer adsorption on solid surfaces. They observed that only a fraction of the polymer is adsorbed and concluded that the amount of adsorption depends on the interaction between the polymer and the solvent as well as on the interaction between the solvent and the solid adsorbent. Adsorption is high from poor solvent as compared from good solvents. The nature of the surface of the adsorbent is also found to affect the rate of adsorption. Information about rates of adsorption and unfolding of protein molecules at the liquid/solid interface is less extensive.

Bull (1956) found that the adsorption of bovine serum albumin at concentrations of 0.037 and 0.185% was nearly complete after 5 mins. He believes that the rate of protein unfolding following adsorption on a solid surface with fixed binding sites might be much less than the rate at the air/water or oil/water interface.

Sorbo (1961) studied the effect of charges on adsorption of proteins on solids. Maximum adsorption of egg albumin, methmyoglobin, oxyhaemoglobin and lysozyme on calcium phosphate gel surfaces was found to take place at their isoelectric pH. Zittle (1953) reviewed the effects of prior adsorption-desorption on the activity of enzymes using chromatography. He adsorbed bacterial amylase, proteases and pectinases on whiting (a pulverized chalk). He stated that "there seems to be no instance in which an eluted enzyme was shown to be inactive". Clauser (1954) also studied various proteins including ribonuclease and hypophyseal growth hormone and have reported no loss of activity after repeated adsorption and elutions on cellulose columns. However, McLaren (1963) who briefly reviewed the kinetics of enzymic reactions at solid surfaces, found that adsorption of enzymes onto solid surfaces in some cases did reduce the catalytic activity. An important reason for differences in enzymic activity measured in bulk solution and at solid/water interfaces is the distribution of ions near the surface which are generally charged. From the adsorption isotherms of serum albumin and γ -globulin obtained by Bull (1957), Orveskes *et al.* (1961), and Hlady *et al.* (1979) the existence of two different modes of adsorption has been suggested. One operating at low, the other at higher protein concentration, respectively. The resulting phenomenon at the lower concentration mode occurs due to random independent adsorption while the higher concentration mode is due to cooperative adsorption.

Morrissey *et al.* (1976) attempted to predict changes in protein adsorption at solid/liquid interfaces by analogy with models of flexible coil molecules. They also showed that two different modes of

adsorption exist. One at low surface coverage where conformational flattening of the molecule occurs and the second at high surface coverage. Here the protein adsorbs in a more compact apparently native configuration. Unlike the situation for flexible polymer chains the conformational transition in protein adsorption on solid surfaces is discrete rather than gradual. Their model predicts that multilayer adsorption is rare. Further Morrissey & Han (1976) suggested that initially adsorbed molecules of protein do not exhibit conformational adjustments to permit the accommodation of additional molecules. This showed that initial adsorption is irreversible.

This result is consistent with work by Absolom *et al.* (1984) who found, from adsorption isotherms, electrophoretic mobility and diffusion rates, adsorption of BSA molecules on glass is also bimodal. The molecules are oriented vertically and have alternating polarities. Electrostatic attraction exists between the protein molecule which are partly dehydrated on adsorption. However, the influence of pH has not been discussed although this could be expected to influence the conformation.

Adsorption of albumin and fibrinogen on cuprophane (regenerated cellulose), silastic (silicone rubber) and Shiley heart valve zeno-grafts also indicate that adsorption is a rapid and an irreversible process (Bornzin *et al.* 1982). The activation energy for the adsorption process was estimated to be about 3kcal/mole for fibrinogen and 6kcal/mol for albumin. Enthalpies for adsorption were very low, approximately 0.4kcal/mole for fibrinogen and approximately 1.5kcal/mole for albumin. Such low enthalpies of adsorption suggested to the authors that there was little or no denaturation of the blood proteins during the adsorption process.

Brash *et al.* (1969) have studied protein adsorption in both static and flow systems on uncharged hydrophobic polymer surfaces. They showed that adsorption is not reversible and the proteins are not desorbed over a wide pH range nor are they removed by extensive ultrasonic vibration. These observations suggest that proteins are uncoiled and strongly bonded to the surface through multiple contacts. Alternatively a layered structure consisting of a surface-denatured layer and alternative layer exists. Such irreversibility has also been observed in the albumin/glass system by Bull (1956).

2.3.3 Adsorption at liquid/liquid interfaces

Though adsorption of proteins onto solid surfaces has been studied by several workers there is little or no progress in understanding the manner in which the implant surface affects the composition of an adsorbed protein layer. An important parameter for determining the activity of the adsorbed protein is the estimation of its interfacial free energy. There is no direct method available for such measurement at solid/liquid interfaces. However, such changes can be determined directly at air/liquid and liquid/liquid interfaces.

Although numerous investigations have been reported for various properties of polymers and protein films, adsorption at air/liquid and liquid/liquid phase boundaries have received relatively little attention. Harowitz *et al.* (1955) suggested that when adsorption at liquid/liquid interface occurs at high film pressures then proteins do not unfold. They observed that serum proteins were present as a unimolecular layer of globular molecules with their long axes parallel to a xylene/water interface when adsorption took place at film pressures

of 12-27 dynes.cm⁻¹. Cumper *et al.* (1950) showed that surface aging was also exhibited by films at liquid/liquid interfaces. They found that pepsin at white oil/water interface formed a layer 7Å thick after 4 min. Depending upon the temperature and the ionic strength the film thickened to about 15Å after 1 hr. Films of low-density lipoproteins isolated centrifugally from human serum or hen's egg yolk also exhibited aging at n-heptane/aqueous solution interface.

It was Fukawa *et al.* (1961) when working with PVA (polyvinyl alcohol) at the air/water interface who observed that changes in the surface tension value occurred gradually over several hours. He attributed this to slow reorientation or polymer adsorption at liquid boundaries. Therefore, certain existing techniques for surface tension measurements were unsuitable as they did not allow sufficient time for equilibrium to be attained. Among these techniques is the drop weight method favoured by many workers (Matthews, 1939; Glass 1968; Lankveld *et al.* 1972) who studied PVA at the paraffin/ water interface and also observed the prolonged times required for equilibrium to be attained and suggested that they were due to diffusion in the case of low concentrations and to conformational changes for higher concentrations. Proteins like polymers also have low diffusion constants in dilute solution. MacRitchie *et al.* (1963) confirmed that the initial decrease of surface tension of proteins is diffusion controlled but suggested that in the later stages, when an appreciable film pressure builds up, the rate of adsorption becomes much slower. This is due to the existence of a barrier which develops as the films become more densely packed. From studies on a number of proteins of various shapes and sizes they explained the role of

pressure barriers in the adsorption process. Using the theory developed by Ward *et al.* (1946), MacRitchie *et al.* (1963) verified that only a small portion of the molecule ($100-175\text{\AA}^3$) needs to penetrate the surface before complete unfolding of the molecule begins. Hence adsorption takes place in three stages. These are: diffusion of native protein molecules to the interface, penetration of the interface by these molecules and finally rearrangement of adsorbed molecules so as to achieve a state of minimum free energy.

2.4 FACTORS AFFECTING THE RATE OF ADSORPTION

The adsorption of macromolecules, their final conformation and the degree of intermolecular interaction depend on many factors. These include concentration, molecular weight, solvent quality, flexibility and electrical charge of the molecule and the properties of the interface.

Adsorption was studied as a function of pH by MacRitchie *et al.* (1963). They found that the net rate of collision of molecules with the surface depended upon the pH, being a maximum at isoelectric point and decreasing on either side. They tried to explain the net rate of collision of molecules by the presence of an electrical potential barrier at the surface and related the rates of adsorption to the corresponding zeta potential.

Also from a study by Kiosseoglou *et al.* (1983) on the interfacial properties of lipoproteins, it has been shown that the time dependent reduction of interfacial tension is influenced by various factors. These are the pH, concentrations of NaCl or methanol and the temperature. The degree of micellar breakdown and subsequent molecular

unfolding and rearrangement following adsorption of lipoprotein molecules increases with decreasing pH and increasing temperature or added methanol.

2.5 CONFORMATIONAL CHANGES OF ADSORBED PROTEIN FILMS

The study of a random coil protein having a flexible structure (casein) and globular proteins with rigid structures (β -lactoglobulin and α -lactoglobulin) by Rahman & Sherman (1982) shows that non-globular protein films expand more than globular proteins in the presence of glyceride mixtures. The casein have extensive hydrophobic regions. The residues involved in hydrophobic bonding are proline, valine, isoleucine, phenylalanine and tryptophan. The globular protein molecules are significantly less hydrophobic than the caseins. Furthermore, globular proteins adsorb more slowly at an interface than random coil proteins. It has been demonstrated by Yudenfreund *et al.* (1975) that the tertiary structure of the globular protein gliadin is disrupted by various surfactants so that it is able to unfold and expand. The covalent disulphide bond is the strongest bond involved in maintaining the tertiary structure of globular proteins. Unfolding of the molecules increases the available number of residues.

Hence adsorption of proteins is an interesting but a complicated process. These macromolecules because of their amphipathic nature display exceptional adsorptive properties as they are highly surface active.

In solution a globular protein molecule, being large and flexible, tends to look like a tangled helix. Its helical character depends mostly on hydrogen bonding along the backbone and the tangled

character more on hydrophobic groups irregularly placed between the amino acid residues. The non-polar groups tend to protect each other from the surrounding water by finding a stable location in the heart of the tangle with the polar amino acid groups arranged around them. Normally adsorption of proteins involves both the hydrophilic and the hydrophobic groups. In such cases adsorbed proteins cannot be easily removed. However, if the adsorption is only due to the hydrophobic bonds, as when adsorbed on paraffin, then the proteins can be easily lifted off.

For a long time it was assumed that when protein molecules are adsorbed at an air/water or oil/water interface, complete unfolding of the molecule occurs. From the limiting area obtained from pressure area isotherms which were $17\text{\AA}^2$ per residue, it was assumed that the polypeptides are organised in extended chains on the surface such that the hydrophobic groups are pointing towards the air or oil at the air/water and oil/water interface respectively, and the hydrophilic groups towards the water. However, on the basis of energy considerations Bamford *et al.* (1956) found the extended chain model unconvincing and suggested that the α -helix structure in the spreading solution retained the same conformation even after removal from the surface. He carried out further electron diffraction measurements on the same samples and found that in all cases the outermost reflections, despite the individual differences, corresponded to a spacing of $1.495 \pm .005\text{\AA}$ characteristic of the α -helix. Additional evidence for the retention of the α -helix conformation was obtained by Loeb *et al.* (1968). They deposited, by the Langmuir Blodgett method, monolayers of poly- γ -methyl-L-glutamate at different

pressures (4, 15 and 27 dynes/cm) on a germanium prism and carried out IR spectroscopy by the multiple internal reflection method - also called attenuated total reflection (ATR). The infrared spectra thus obtained for monolayers deposited at any constant pressure established that the polypeptides retained their α -helical conformation in the monolayer.

However, a change of secondary structure in the monolayer does occur although it is influenced by the stability of the bulk conformation. This was further confirmed when Loeb (1969) compared the surface conformation of paramyosin (one of the most stable proteins of helical nature) with β -lactoglobulin. He found that the structure of β -lactoglobulin which, in the bulk, is less helical and less stable is more susceptible to a conformational transition in the surface. As the secondary structure demonstrated a high inertia to change the surface denaturation has to be mainly due to the changes in the tertiary structure. Also the tertiary structure is found to be more susceptible to permanent modification by surface forces than the secondary structure. In order to rupture an α -helix by adsorption forces, the standard free energy per residue has to be very high but it is seen that even a slight increase in the standard free energy will cause some change in the conformation of a molecule. Therefore when considering conformational changes caused by surface forces, major attention should be given to the tertiary structure. Even if the secondary structure is ultimately affected, this can occur only after rupture of the tertiary structure. The degree of conformational change accompanying changes in the tertiary structure observed is lower in solutions of high concentration. It is the tertiary struc-

ture which is stabilised by intermolecular interactions at high surface concentrations.

2.6 BEHAVIOUR OF ADSORBED HUMAN SERUM ALBUMIN AT AN INTERFACE

To understand the behaviour of the HSA molecule at an interface it is essential to consider its structure at three different levels. The primary structure is the number and sequence of the amino acid in the polypeptide. Its conformational rigidity arises from disulphide bridging. In the amino acid sequence there are 17 cys-cys pairs but because of the rigidity they cannot form bonds between themselves but have to bridge with isolated cysteine groups.

The secondary structure of albumin contains 50-55% α -helix, about 15% β -pleated sheet, and the remainder random coil. The S-S bridges tend to stabilize the secondary structure by retaining the α -helical form as they tend to impose considerable restraints. This was shown by Reed *et al.* (1975). They studied 12 isolated fragments of BSA. The 11 fragments containing S-S bridges retained the predicted amount of α -helix but the 12th fragment, which lacked the disulphide bonds, did not.

The molecule of serum albumin has a relatively low content of hydroxy amino acids compared with other proteins. The OH^- side chains form hydrogen bonds with side chains containing -COO^- or =NH^+ groups. If the content of hydroxy amino acid is high then cross links will be formed with all the available -COO^- and =NH^+ groups giving the molecule a rigid structure as all =NH^+ would be bound internally. However as few hydroxy amino acids are available in the albumin molecule, all the =NH^+ groups are not bonded leading to a molecule

which is not very rigid. Hence as the pH of the environment is changed the molecule binds with the available H^+ or OH^- molecules which causes a change in its configuration.

The tertiary structure represents the amount of coiling and folding of the polypeptide required to attain the globular shape as a prolate ellipsoid within major and minor axes of $140\text{\AA}$ and $40\text{\AA}$. This shows that the strong associative forces which exist between some of the loops are responsible for the observed compact molecule. More than 200 positive and negative charges which are distributed over the molecule give albumin an hydrophillic character and contribute to its high solubility in aqueous media. As a result protein molecules in aqueous solution have a relatively hydrophobic interior and a relatively hydrophillic periphery, but because of its amphiphillic composition it tends to be surface active. However, on adsorption structural changes in the molecule are induced by the interface at the expense of intramolecular hydrophobic interactions. Therefore at air/water or oil/water interfaces the structural changes in the molecule minimizes the hydrophobic interaction by causing the non-polar groups to protrude into the air or oil phase.

The degree of stability of the tertiary structure of different proteins was examined by Cheesman *et al.* (1954), Llopis *et al.* (1960), Ray *et al.* (1956), Yamashita *et al.* (1967) and Evans *et al.* (1970). By comparison of surface pressure-area ($\pi:A$) curves with surface pressure-concentration ($\pi:c$) curves Evans *et al.* (1970) studied the surface behaviour of β -casein, bovine serum albumin (BSA) and lysozyme and concluded that the factors which control the structural stability of these proteins in the surface are the hydrophobicity and the

strength of the forces maintaining the tertiary structure. The concentration of S-S bonds is an important factor but not the sole one in preserving the tertiary structures. BSA with 17 S-S bonds per molecule and a molecular weight of 66,000-69,000 has almost the same number of S-S bonds as a lysozyme molecule. However, it is considerably less resistant to denaturation (Foster 1960) because lysozyme, according to the hydrophobicity scale derived by Bigelow (1967) based on the work of Tanford (1962), is about 15% more hydrophobic than BSA. β -casein is found to be very flexible as it is even more hydrophobic than BSA and also lacks S-S bonds to fix the tertiary structure. Camejo *et al.* (1968) suggested that high hydrophobicity and flexibility were also the reasons for the marked surface activity of high density apolipo-protein.

As the pH of the solution changes, the distribution of free and bound charges around the protein molecule alters. The field causes stresses in the molecule resulting in changes in spacing of the interior groups. The structural changes will be greater the more the pH differs from the isoelectric point of the protein. Hence, at high and low values of the pH the forces stabilizing the tertiary structures are lower, the chains have greater rotational mobility and unfolding becomes easier.

2.7 TYPES OF PROTEIN FILMS

Extensive studies of protein films at interfaces have led to the elucidation of their film forming properties. Most of the available work concerns films spread from solution on to the surface of a liquid. Very little reliable work is available for films adsorbed

from solution as proteins take a long time to reach a steady state which is necessary for obtaining reproducible results. Very few workers left the films for a sufficiently long time.

The earliest method of obtaining protein films was used by Devaux (1904), Metcalf (1905) and Keenan (1929). It consists of dispersing the proteins in water or dilute alkali and dropping a small quantity on the surface of the liquid. From the maximum spread area obtained Devaux estimated the thickness of the film to be 30-80Å for albumin, peptone and casein. Metcalfe obtained 30Å as the minimum thickness of a solid film of peptone whilst Keenan obtained for gelatine on mercury at pH4.8 a thickness of only 7.5Å. However, this method was considered to be unsuitable as it caused hydrolysis of the proteins and the film formed was not homogeneous, therefore attempts were made to form films by direct spreading. This was achieved in two ways: (1) by adding spreading agents such as isopropyl alcohol which helps to spread the applied drop of protein solution over the surface of the bulk, usually an aqueous solution; and (2) by spreading a thin film first formed by pouring a solution of the protein via a glass slide or glass rod on to the surface in an effort to eliminate the possibility of the protein entering the bulk phase.

It has been shown by Trunnit (1960) that the method of spreading the protein is crucial to the properties of the protein film because unknown amounts of protein may dissolve during the spreading. As mentioned earlier, the author's own experience with this technique has shown that the surface tension outside the barriers confining the spread film, decreases indicating that material transfer has taken place through the bulk phase. This shows that spread films differ

appreciably from adsorbed films of the same protein. However, some workers (Cumper *et al.* 1950; MacRitchie *et al.* 1961; and Benhamon *et al.* 1960) have found evidence that spread protein monolayers and adsorbed protein films are virtually identical but James *et al.* (1964) have shown that this assumption may be valid only if the comparison is restricted to concentrated spread monolayers in which there are some native protein molecules present. In this situation the rate of protein adsorption is likely to be as fast as spreading of the monolayer and so the unfolding of the molecules at the two surfaces is likely to be similar. Ghosh *et al.* (1963) have also supported the fact that the films obtained by the spreading technique and adsorption would be similar but only when solutions are concentrated.

It is clear from the above that a study of adsorbed protein films examined under carefully controlled conditions would help in our understanding of the nature of the changes which occur as the surface concentration builds up until the film is in equilibrium with the bulk phase.

CHAPTER THREE

SURFACE AND INTERFACIAL TENSION MEASUREMENTS

3.1 INTRODUCTION

What is meant by an interface? A general definition of matter commonly refers to bulk properties, that is, matter as it is found in appreciable mass and volume with little or no attention being given to various surface phenomena. In recent years the great significance of the surface properties for many systems has been appreciated.

It is well-known that matter does not exist in the interior of the sample under the same conditions as it does near the surface. Surface reactions are found to have great importance in nature, in general, and particularly in the domain of biology. In the interpretation of surface phenomena, the general problem is to determine the properties of a system which are due to interaction of molecules at the surface.

Therefore, a surface is defined as the boundary of a phase which refers to an homogeneous mass of a solid, liquid or gas. When two phases are in mutual contact the boundary that exists between them is called an interface. Strictly speaking, the surfaces commonly referred to as liquid and solid surfaces should be considered as liquid/air and solid/air interfaces, respectively. The different types of interface that exist due to the three states of matter can be referred to in a general way as gas/liquid; gas/solid, liquid/liquid, liquid/solid and solid/solid interfaces.

It is difficult to give exact dimensions to an interface as it is not a distinct entity in the mathematical sense which separates two

phases, but is rather a region of small but finite thickness and has measurable properties differing from those of the material in the homogeneous phases.

The mathematical treatment of surfaces was developed much earlier than the development of accurate methods for the measurement of interfacial tension. These methods are primarily based upon the tendency of a finite volume of a liquid to adopt a minimum surface area when unconstrained. The sphere is known to possess minimum surface area for a given volume and this is also the shape assumed by any free body of liquid. But if the surface is partly constrained then it will adopt an area consistent with the constraint.

3.2 STABILITY OF THE INTERFACE

All forms of matter, solid, liquid and gaseous, are made up of molecules which are subjected to various intermolecular forces. Consider a molecule in a liquid. If it is lying in the bulk it will be surrounded by other molecules and the intermolecular forces experienced by that molecule on the whole will be balanced. However a molecule at the surface will be subjected to imbalanced forces due to the attraction from the interior of the liquid. This results in the density of the surface layer being lower than the bulk. Hence the surface is in a higher energy state and has a tendency to contract. The force of contraction per unit length that the molecule at the surface experiences is known as the surface tension, γ .

At the interface between two liquids there is again an imbalance of intermolecular forces but of a lesser magnitude. In the absence of orientation effects liquid/liquid interfacial tension, γ , usually

lies between the individual surface tensions of the two liquids at the interface.

A certain amount of work is required to bring the molecules from the bulk to the surface. This work per unit area is also referred to as the excess surface free energy,

$$\gamma_o = \frac{dw_o}{dA} \quad 3.1$$

where γ_o is expressed in mJm^{-2} ,

w_o is the work done,

and A is the area.

It is essential for the interfacial surface free energy to be positive for the stable existence of an interface because if it were either negative or zero, then the movement of molecules could cause the surface region to expand continuously which would lead to a complete dispersion of one material into the other. These conditions where no opposition is offered to the dispersive forces occurs between two gases or between two miscible liquids. However dispersion can occur in two immiscible liquids when the interfacial energy is reduced by the presence of another component. This is commonly known as spontaneous emulsification.

3.3 MOLECULAR ORIENTATION AT INTERFACES

The free energy per unit area of a pure liquid cannot be altered except by orienting the surface molecules so that the ends with the largest field of force point inwards. In the case of solutions of two or more substances, whose molecules differ in the intensity of

their fields of attractive forces, the surface free energy can be diminished in a different way. The molecules which, comparatively, have greater fields of force tend to pass into the interior. The surface layers of the solution will therefore have a higher concentration of the constituent whose intrinsic free energy is small. This tendency of the molecules of one constituent of a solution to partition at a surface is called adsorption. Positive adsorption of a constituent is an increase of concentration at a surface; negative adsorption a decrease.

Qualitatively, the general rule for adsorption is that if the solution has a smaller surface tension than the solvent, the solute is concentrated at the surface, if it has a larger tension the solute is driven as far as possible into the interior. It is possible to obtain very considerable decreases in surface and interfacial tension by adsorption of a substance which has itself a very low surface tension. In such cases the adsorption may proceed so far that the surface layer consists almost entirely of the molecules of the constituent which has the smaller field of force. Large increases in surface tension cannot, however, be obtained in a solution by using solutes with surface tension much greater than the solvent. Such solutes are driven into the interior, and the surface layer tends to approximate to the pure solvent containing few of the molecules with a large field of force. Consequently the surface tension is raised but little above that of the solvent.

Organic substances containing more than two carbon atoms in the molecule usually show positive adsorption in water. It is seen that as the length of the hydrocarbon chain increases adsorption also increases and surface tension is reduced.

Adsorption of surface active hydrocarbons from aqueous solutions lowers the surface tension to about 25 dynes cm^{-1} (Adamson, 1976) which is almost equivalent to the surface tension of the paraffins. The surface layer then consists almost entirely of long, saturated hydrocarbon chains. According to Traube's rule (Traube, 1891), for each additional CH_2 group in a homologous series of surface active substances the concentration required to give a certain surface tension is reduced by a factor of 3. Inorganic salts are usually negatively adsorbed, and the tension may be raised in strong solutions but only by a few dynes cm^{-1} . The quantitative relation between the amount of adsorption and the change in surface tension was first deduced from thermodynamic considerations by Gibbs in 1931. The significance of his work for protein adsorption will be discussed in more detail in Chapter Four.

Adsorption of surface active material on various surfaces is of great importance in many biological and technological processes. Typical examples are in general detergency, the production of stable emulsions, the function and structure of cell membrane and the development of suitable substances for use as prostheses and for the slow release of drugs. It is not only the number of molecules adsorbed at an interface that is of ultimate importance but also the changes in mechanical properties such as the resistance of the interface, to deformation, that occur as a result of the adsorption. Hence adsorption is one of the characteristic surface properties which play an important part in determining the physical property of a system as a whole. Therefore it is of prime interest to elucidate the characteristics of proteins at different interfaces although determination of

function and structure of protein is found to be far more difficult at interfaces than in the bulk solution.

Macromolecules occupy a special position with regard to surface effects. For the same amount of material in the surface less work has to be done against the concentration difference since due to their large molecular weight fewer molecules have to be transported, as energetic interactions with the surface are due to mass effects which remain the same. Therefore macromolecules adsorb far more readily than their low molecular weight counterparts.

In the case of liquid/gas and liquid/liquid phase boundaries the interface is essentially a flat, very narrow, two-dimensional transition zone where the fraction of surface occupied and the surface free energy change (or surface tension) depends on the energetic effects of bringing a polymer segment into the surface but the total amount adsorbed and the thickness of the adsorbed layer depends to a large extent on the solvent and on the flexibility of the molecule. As surface layers have important implications in many phenomena and it is known that in the case of adsorption from solution the outermost layer determines the surface tension, it is therefore vital that the surface tension of the solution should be accurately determined. The mechanical properties of the adsorbed film will depend on the configurational freedom of the molecules. Therefore it is essential to determine the conformation adopted by the molecule with changes in surface concentration. Methods for the measurement of surface tension were invented during the last century by Gibbs and Wilhelmy and since then innumerable methods have been developed.

3.4 REVIEW OF METHODS FOR INTERFACIAL TENSION MEASUREMENTMENTS

As there are a great variety of methods available for the measurements of interfacial tension, these techniques are briefly mentioned here in order to explain the reason why the particular technique used for this work was chosen.

The techniques are broadly divided into three groups depending on the parameter they use for determining the interfacial tension.

1. Procedures using pressure methods.
2. Procedures involving detachment methods.
3. Techniques using shape determinations.

3.4.1 Methods based on change of pressure for curved interfaces

The principal methods in this class are:

1. capillary rise method;
2. maximum drop or bubble pressure method.

3.4.1.1 Capillary rise method: This is a simple method and is found to be very accurate for pure liquids but has difficulties when used with protein solutions. Problems arise mainly as a result of deposition of protein material on the wall of the tube. This causes uncertainty regarding the value of the contact angle which has to be determined. It also requires the exact knowledge of the density of the liquid and the radius of the capillary tube. Also it is rather difficult to obtain cylindrical tubes with constant bore.

3.4.1.2 Maximum drop or bubble pressure methods: Though suitable for pure liquids they are not accurate when complex surface active materials are being used. In the case of bubbles, the surface created will in general be only a fraction of a second old and therefore will not be an equilibrium surface, especially for materials such as proteins which require many hours to reach equilibrium. Moreover, in the case of drops, difficulties arise in maintaining saturation conditions for the duration of time involved and also the aging of the surface causes the γ -values to reduce and for small drops this induces instability.

3.4.2 Methods involving detachment

In all these methods the surface of the liquid in contact with the sensor is expanded as the point of detachment is approached. This means that the liquid surface will not normally maintain its equilibrium during this time. In general this condition can only be met when diffusion of the surface active molecules to the surface is very fast.

Different detachment methods normally used are:

1. Du Nouy ring method.
2. Wilhelmy Plate method and detaching cone method.
3. Drop volume and drop weight method.

3.4.2.1 Du Nouy ring method: This method is frequently used for pure liquids but it is not highly dependable for reproducibility in determining surface aging effects for protein solutions which take a long time to reach equilibrium.

3.4.2.2 Detaching cone method and Wilhelmy Plate method: In these methods, measurements are made using the cone and the plate respectively. In principle cone and plate can be used both as a detachment method or as a static method. When used in the detachment mode they tend to give higher values which are of the newly created surface rather than its equilibrium value. Such uncertainties can be avoided if the equipment is used in a static mode, but here it is essential that complete wettability of the plate or cone is achieved. Accurate and more precise measurements are then obtained. Hence the Wilhelmy Plate method, which will be explained in detail later in this chapter, has been used in combination with the surface balance for determining the changes in the surface tension values of protein solution of different concentration at both air-liquid and liquid-liquid interfaces.

3.4.2.3 Drop volume and drop weight method: This detachment method is not considered very reliable. Here either the weight or volume of the drop detaching from a narrow tube is determined. The top of the tube should be completely wetted or a sharp edge tip has to be used. It is seen that on detachment the complete drop does not leave the tube. For accurate measurements the equipment has to be free from vibrations and the last 10% of the drop should be formed very slowly. Also a proper correction factor has to be introduced.

3.4.3 Techniques using shape determinations

Methods depending on shape determination are:

1. Pendant drop method.
2. Sessile drop or bubble method.
3. Spinning drop method.

3.4.3.1 Pendant drop method: Preliminary experiments using the shape of pendant drops were carried out to determine the surface aging of protein solutions. The method, which is described in detail later, involves photographing the drop at various time intervals as the surface comes to equilibrium. From the photographic data and using correction tables, the surface tension is evaluated. The basic disadvantage associated with the shape determination method is that significant time is required initially to set up the drop during which no information can be recorded. Therefore, from these experiments it is only possible to determine the order of magnitude of the time taken for the surface to reach equilibrium.

3.4.3.2 Sessile drop and bubble method: In principle the shape of a sessile drop or bubble could be used to determine the surface tension of a protein solution. The disadvantages associated with the method when compared with other techniques is that being a shape determination method, initial changes cannot be obtained and also the surface area involved cannot be changed in a controlled manner. For these reasons the method was rejected.

3.4.3.3 Spinning drop method: In this technique measurements are made when the length of the spinning drop is at least 4 times its static diameter. This technique is most suitable for determining very low interfacial tension. It can be used to measure values around 0.001 mNm^{-1} easily and accurately.

3.4.4 Dynamic methods: When initial information regarding the aging processes occurring at the surfaces are required, a dynamic method must be used. The attainment of the equilibrium values of the surface-tension occurs as the result of a series of relaxation processes.

First the molecules diffuse from the bulk and adsorption takes place then the molecules undergo orientation and conformational changes. The rate at which the various relaxation process takes place determines the time taken for the surface to come to equilibrium. A study of dynamic effects yields greater insight into these molecular processes. Existing dynamic methods are:

- 1) The damping of surface waves.
- 2) Oscillating liquid jets.
- 3) Dilating surface techniques.

3.4.4.1 The damping of surface wave: The properties of waves have received great attention in recent years due to their importance in surface films for many technological processes. The mechanical properties of many disperse systems depend on the same dynamic surface properties that govern the propagation of surface waves. Damping which is the loss of kinetic energy of the waves

depends on the viscous friction of the liquid below the surface. So an increase in viscosity would increase the damping effect. However variations in damping are also produced when surface active material is adsorbed at the surface. This surface tends to resist the expansion and compression caused by the propagation of waves. This creates a flow pattern in the liquid below the surface resulting in an increased viscous friction which enhances the damping effect.

3.4.4.2 Oscillating jet method: The fluid under investigation issues from an ellipsoidal orifice at high velocity. As a result of surface tension forces the elliptical cross-section relaxes and an oscillation is set up resulting in the jet having a wave-like envelope. Very fast relaxation processes can be detected using this method but on the other hand it is quite unsuitable for processes having long relaxation times.

3.4.4.3 Dilating surface technique: Two main forms of this technique exist. The first involves measuring the pressure in a small drop. The surface area of the drop is expanded or contracted by the addition or subtraction, respectively, of some liquid from the drop. The decay of surface tension is obtained from changes in pressure which follow the sudden change in area. In the second technique the surface film under investigation is confined between barriers on a Langmuir trough. Expansion or contraction of surface film is affected by movement of one or both barriers. Here the changes in surface tension are recorded

using the Wilhelmy Plate technique. Both of these techniques were used in the present study and will be described in more detail in the following sections.

3.5 EXPERIMENTAL TECHNIQUES USED TO MEASURE THE SURFACE TENSION OF HUMAN SERUM ALBUMIN (HSA)

In the investigation carried out here the static and dynamic interfacial tension measurements were made in order to gain a better understanding of the adsorption characteristics of protein and the conformation changes following it.

The pendant drop technique was employed to make the initial measurements. This technique was chosen because, besides being widely used and simple to operate, it was possible to control the size of the drop. The measurements thus obtained gave reproducible results. In addition only small amounts of materials under investigation were required.

At first, estimates of the surface aging of the adsorbed protein films were made. From the results obtained it was seen that several hours were needed for them to reach equilibrium. However, rapid changes in the surface tension occurred only during the first hour, after which the changes were more gradual. Some problems were experienced in determining the initial changes. These were attributed to operational difficulties, in particular the time taken to set up the drop and allow mechanical vibrations to subside meant that much of the information about the initial changes was lost. Because of interest in these rapid changes and in order to detect the small changes which occur as equilibrium is approached more sensitive dynamic techniques

have been used, one of them being the Wilhelmy Plate technique combined with a surface balance. This was modified to enable time dependent measurements to be made easily and rapidly. Hence like other dynamic methods surface aging and relaxation effects for very small time scales could be studied. But the advantage it had was that it enabled measurements to be made for longer periods, up to several hours.

The other technique used was the dilating drop technique. By this method determined the rapid initial changes that occurred when the area of an equilibrated drop was suddenly changed. The advantage of this technique was that only small amounts of the solution were required and gave good reproducible results, once humidity and vibration isolation conditions were controlled. All the equipment used for this work was developed and built at Imperial College. The apparatus for the pendant drop technique and the Wilhelmy Plate technique are described in detail in this chapter as these two techniques were initially used to determine the surface aging of protein solutions. However as the dilating drop technique was used mostly for the dynamic measurements therefore the apparatus and mechanism is explained in detail in Chapter Four.

3.5.1 The pendant drop method: This is a static method for the measurement of surface tension. The shape of the drop which is suspended from a fine capillary is controlled by the surface tension forces and gravity. The method was first developed by Bashforth & Adams (1883) and improved by Andreas, Hauser & Tucker (1938).

The schematic diagram of the pendant drop apparatus is shown in Figs. 3.1a and 3.1b. A drop of the liquid under investigation was formed at the tip of an Agla micrometer syringe which was enclosed in a double walled chamber having optical windows. The whole cell was thermostatically controlled. The drop was illuminated by a collimated beam of monochromatic light from a mercury vapour lamp. The equipment was mounted on an anti-vibration table to reduce external vibrations. The profile of the drop is photographed at about 13 times magnification using a suitable camera. A series of photographs are taken at various time intervals over a period of several hours until the shape of the drop remained constant.

The surface tension of the solution forming the drop was calculated from the profile, as shown in Fig. 3.2, using following relationship:

$$\gamma = g\rho \frac{d_e^2}{\kappa} \quad 3.2$$

where g = acceleration due to gravity;

ρ = density of the liquid;

κ = the correction factor;

d_e = the largest diameter of the drop.

The correction factor, κ , depends upon the shape of the drop. Andreas *et al.* (1938) characterised the shape of the drop by the ratio, S , defined as

$$S = \frac{d_s}{d_e} \quad 3.3$$

where d_s is equal to the diameter in a horizontal plane, at height d_e from the apex of the drop as shown in Fig. 3.2.

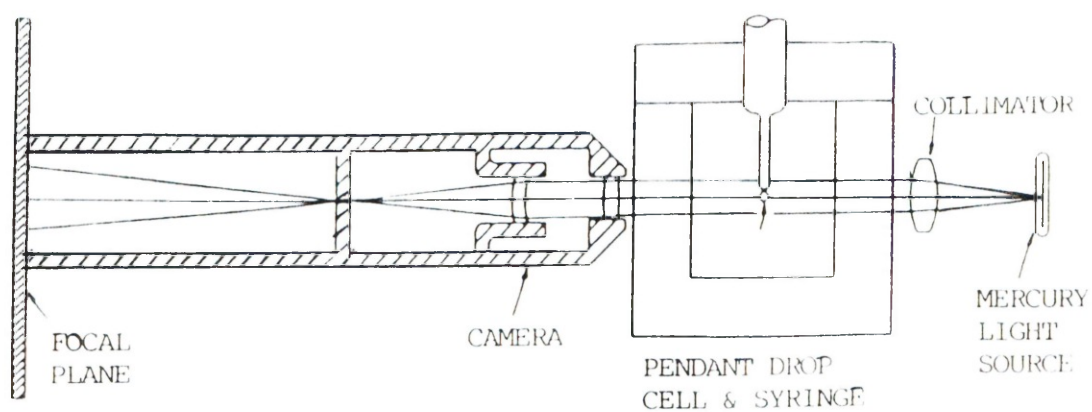


Fig. 3.1a Schematic diagram of the pendant drop apparatus used for the surface tension measurement.

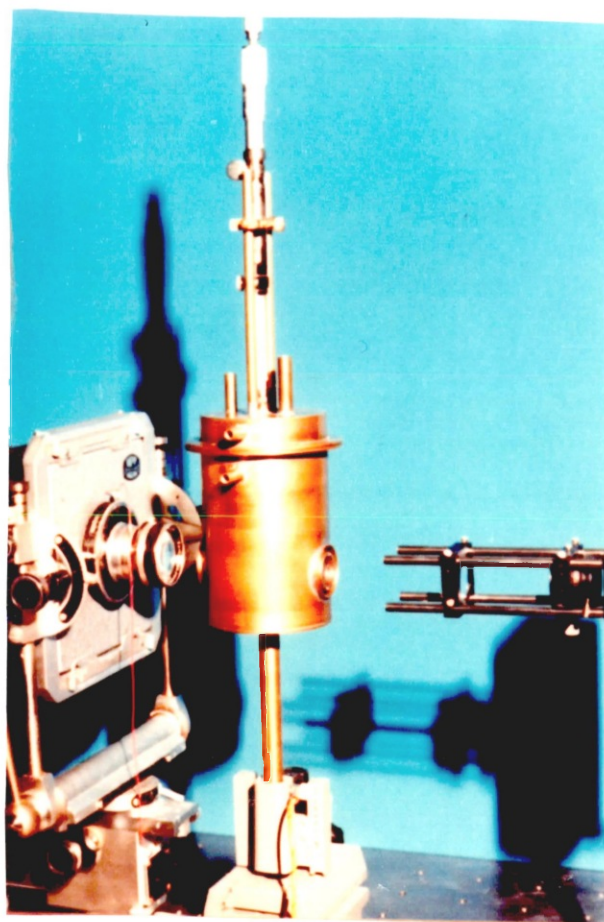


Fig. 3.1b Close view of the thermostated chamber in which the drop is formed and allowed to reach equilibrium.

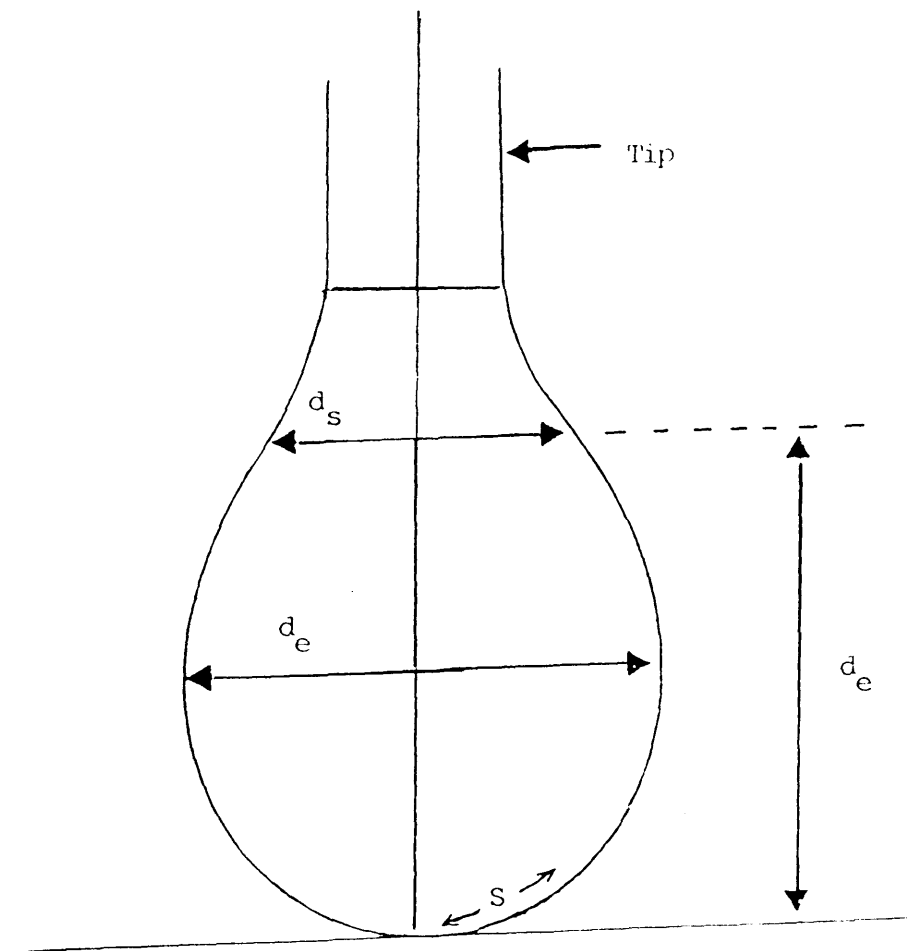


Fig. 3.2 Section of the drop showing d_e - the maximum diameter of the drop and d_s - the diameter measured at height d_e from the apex of the drop.

The values of $1/\kappa$ were tabulated by Andreas *et al.* (1938) as a function of S from known values of the surface tension of water. Later, Fordham (1948) compiled a more accurate table by numerical solution of the fundamental differential equation.

However Roe, Bacchetta & Wong (1967) used several characteristic ratios to define the shape of the drop instead of only one. A series of diameters, d_n , for $n = 8 \dots 12$ was defined and measured at heights Z_n from the base of the drop, where $Z_n = n/10 d_e$. The corresponding characteristic ratios were then given by:

$$S_n = \frac{d_n}{d_e}$$

where $n = 8 \dots 12$

A series of tables giving $1/\kappa_n$ as a function of each S_n was compiled by Roe *et al.* (1967). Hence from a single photograph of a pendent drop as shown in Fig. 3.2, several values of S_n were measured using a microscope at 30x magnification with an accuracy of $\pm 0.5 \mu\text{m}$. From the measurements of S_n the values of $1/\kappa_n$ were calculated. The surface tension was determined from the average value of $1/\kappa_n$ using Eq. 3.2. This method improved the accuracy of the surface tension values thus obtained. A marked variation in $1/\kappa_n$ values was attributed either to an error in the measurement of d_n or to the drop not having attained equilibrium. In the latter case the experiment was repeated.

3.5.2 The Wilhelmy Plate technique

As dynamic measurements were to be made using this technique, it was designed to function in conjunction with a surface balance. The variations in surface pressure with the area available to the surface active material are determined by using a surface balance. In the apparatus used here, which is shown in Figs. 3.3, 3.4 and 3.5, the surface film is confined between two movable barriers, whose motion is accurately controlled by a reversible electric motor, and the surface tension is measured using a Wilhelmy Plate which is attached to a micro balance.

This enables time dependent measurements to be made easily. The trough and the barriers for air/liquid measurements were made of PTFE. As PTFE tends to warp, both the trough and barriers were strengthened using stainless steel plates. PTFE was used in order to ensure a high contact angle and also to allow the level of water to stand above the rim of the trough. The rim of the trough and the barriers were smoothed by polishing on a roughened glass plate, as smooth surfaces ensured good contact thus avoiding leakage. The temperature was controlled by allowing water from a constant temperature water bath to circulate through glass coils placed in the trough.

The trough for liquid/liquid measurements was made of glass in which a Brooks Frame was placed. A new design of the original Brooks frame is used. It consists of two pyrex glass strips held accurately parallel to each other in a stainless steel adjustable block. The glass strips form the rails on which rests the motor driven barriers, used to expand and compress the films adsorbed at an liquid/liquid interface. The surfaces of the rails are made coplanar before use by careful grinding. Parts of the barriers and the rails are rendered

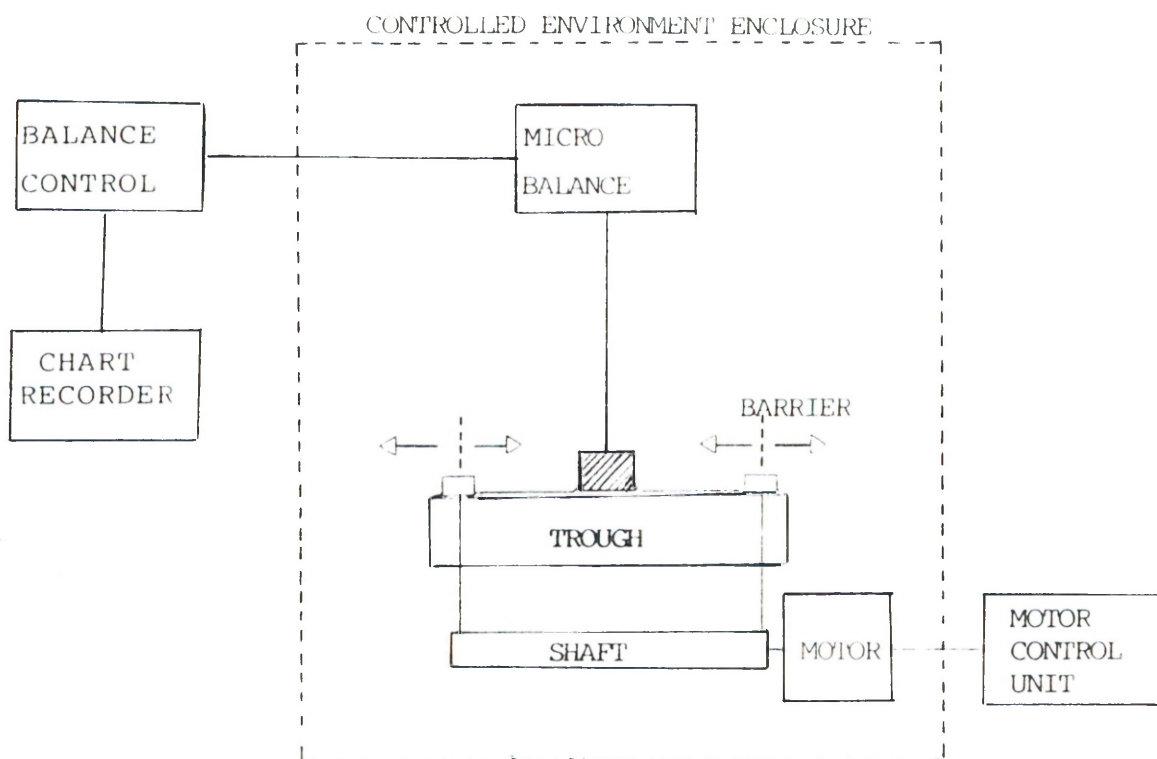


Fig. 3.3 Schematic presentation of the Wilhelmy plate method as used for static and dynamic surface tension measurements.

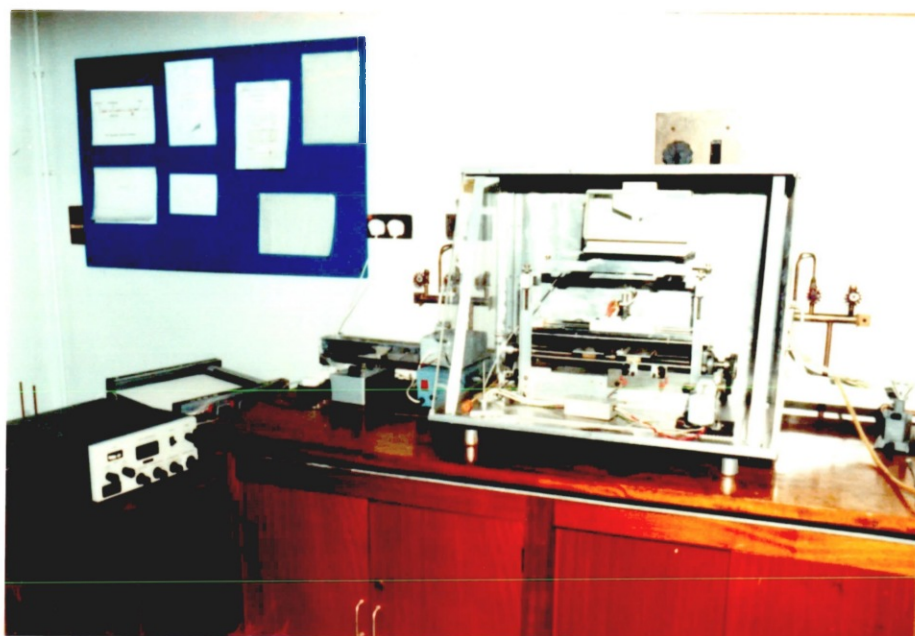


Fig. 3.4 Photograph showing the entire apparatus used for the experimental work.

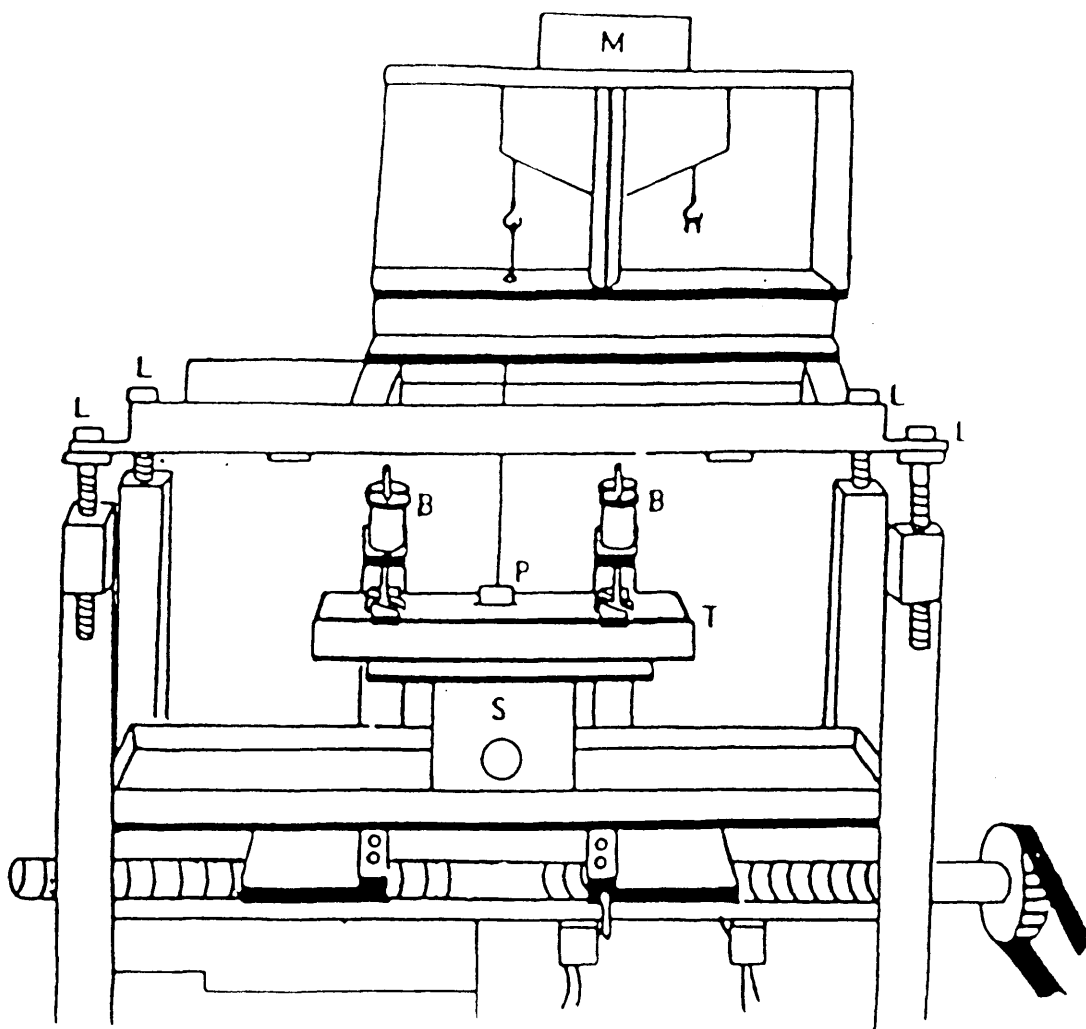


Fig. 3.5 A closer view showing M - microbalance; P - platinum or filter paper plate; B - motor driven barriers; T - Langmuir trough; S - stage for raising and lowering the trough; s - levelling screws for leveling the micro-balance. The micro-balance is connected to a balance control unit and a chart recorder not shown in the figure.

hydrophobic by treatment with dimethyl dichloro silane solution. This is done to prevent the film enclosed between the rails and the barriers from escaping - see Fig. 3.6. The temperature is controlled to within $\pm 1^\circ\text{C}$ by circulating water through copper coils placed in a stainless steel bath outside the trough.

The air/liquid and liquid/liquid interfaces were cleaned by sweeping the movable barrier across the interface. The compressed contamination was removed by suction using a clean glass capillary connected to a water pump. The capillary was appropriately bent at one end to allow easy positioning of the tip at the interface.

The Wilhelmy Plate used was made either of roughened platinum or of filter paper. The advantage of using a paper plate is that zero contact angle was always ensured. The perimeter of the plates used was accurately measured using a low-power microscope. The plate is positioned so that its edge is just immersed in the liquid surface. After the mass of the plate and support has been counter balanced, the additional force on the plate, i.e. force due to the surface film is given by

$$F = \gamma \cdot 2 (l + l_o) \cos \theta_c \quad 3.4$$

or

$$F = \gamma p \cos \theta_c$$

where F = force,

γ = surface tension,

p = perimeter of the plate,

θ_c = contact angle,

l = length of the plate,

l_o = thickness of the plate.

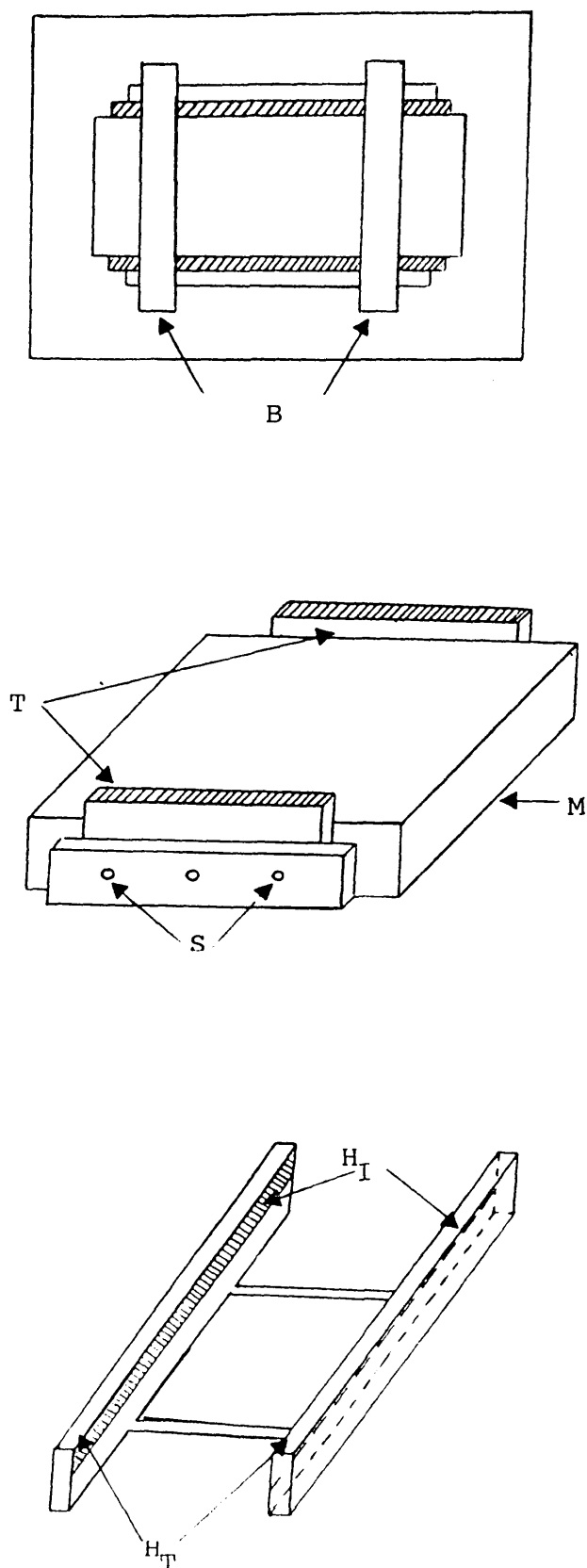


Fig. 3.6 Glass trough with modified form of Brooks frame used at liquid/liquid interfaces. B - movable barriers; s - screws to hold the slides; T - slides forming the trough; M - metal block; H_I - hydrophilic inside edges; H_T - hydrophobic top edges. Top: Trough from above. Middle: Front view of Brooks frame. Bottom: Brooks frame treated with dimehtyl dichlorosilane solution.

If complete wetting of the plate is achieved the contact angle becomes zero and the expression simplifies to

$$F = \gamma \cdot p \quad 3.5$$

Dynamic measurements may be made in two ways using this equipment. Firstly the barriers may be used to expand and compress the film in a regular way using the motor drive or secondly, a sudden expansion or compression may be achieved by a single displacement of the barrier.

To avoid contamination and draught the entire apparatus is enclosed in a perspex box with sliding doors. This enabled proper positioning of the trough.

The balance was calibrated each time prior to an experimental measurement. Using this technique it was possible to measure surface tension with an accuracy of $\pm 0.25\%$.

3.6 MATERIALS

The protein HSA (Cohn Fraction V) which had protein content greater than 95% was obtained from Koch Light Laboratories and used without further purification. For each experiment fresh solutions were prepared in phosphate buffer.

The aqueous substrate used in this study was a phosphate buffer made by dissolving 114 mM NaCl, 18.7 mM disodium hydrogen orthophosphate in pure water to give 156 m equivalent of Na^+ and a pH of 7.4 ± 0.1 as an interstitial fluid.

Pure water was obtained by distillation of deionized water from alkaline potassium permanganate solution under a stream of N_2 gas in an all glass still. For the oil phase n-decane was used. All these chemicals were of Analar 'R' grade and obtained from BDH Chemicals Ltd.

Prior to use all the equipment was thoroughly washed in Decon 90 solution to remove dirt and grease. Then they were soaked in a 5% solution of "RBS concentrate", a cleaning solution, for 6-8 hours. If the apparatus was heavily soiled then a 10% solution was used and the soaking time was increased up to 24 hours. They were then thoroughly washed with deionized water and finally rinsed in pure water.

3.7 RESULTS

3.7.1 Surface Aging

In order to understand the conformational changes that occur in a molecule of protein on adsorption, changes in the interfacial free energy have been studied.

Measurements obtained by the pendant drop method for non-polar liquids are given in Table 3.1 (see over) and compared with literature values to determine the reliability of the apparatus.

As the above values are in good agreement, it is reasonable to state that the apparatus constructed here was free of faults and can be used to perform measurements of HSA at various concentrations. Results are as shown in Fig. 3.7. From the diagram it is seen that as the concentration increases both the initial and final values of

TABLE 3.1

Comparison of the surface tension values obtained using the pendant drop apparatus with the values obtained from the literature. This was done to test the reliability of the constructed pendant drop apparatus. $T = 25^{\circ}\text{C}$.

Liquid	γNm^{-1} (observed value)	γNm^{-1} (Literature value)
n Heptane	19.97	20.14
n Octane	21.70	21.62
Iso Octane	19.58	19.6
n Decane	23.80	23.82

the surface tension decrease. Also from Fig. 3.7 it can be seen that the values apparently become constant after about 30 mins. More sensitive techniques, however, show that small changes continue to occur for many hours. Therefore further experiments were carried out using the Wilhelmy Plate technique.

The reliability of the equipment was checked by studying the behaviour of stearic acid at air/water and air/acid (0.01M HCl) interfaces. From Fig. 3.8 it is seen that the area obtained by extrapolation is in very good agreement with the literature value of

$$A = 0.22 \text{ nm}^2 \text{ mol.}^{-1} \text{ at an air/water interface, and}$$

$$A = 0.25 \text{ nm}^2 \text{ mol.}^{-1} \text{ for stearic acid on HCl.}$$

Hence it was concluded that the apparatus constructed would give reliable results.

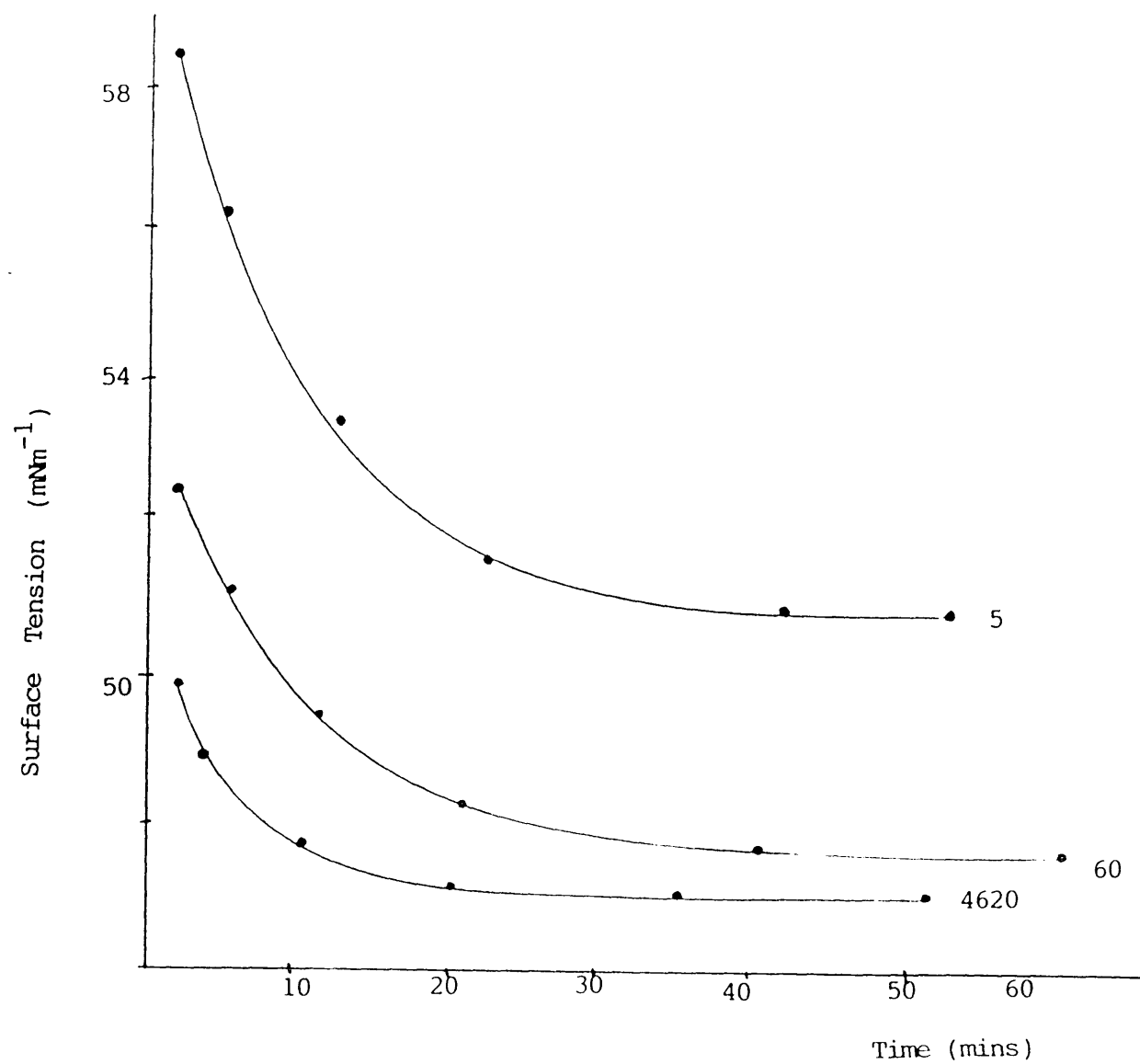


Fig. 3.7 Surface tension values at air/water interface for human serum albumin solution in phosphate buffer. Concentrations shown in mg% at temperature 36°C.

Changes in surface tension values of adsorbed HSA were determined for a period of 17 hrs. These changes were determined at both the air/liquid and liquid/liquid interfaces for pH 3, 4.7, 7.4 and 11. The rate of changes in the surface tension values are shown in Figs. 3.9 and 3.10. Variations in the surface tension values as a function of pH were also studied for different bulk concentration at both the air/liquid and liquid/liquid interfaces. These changes are shown in Figs. 3.11 and 3.12.

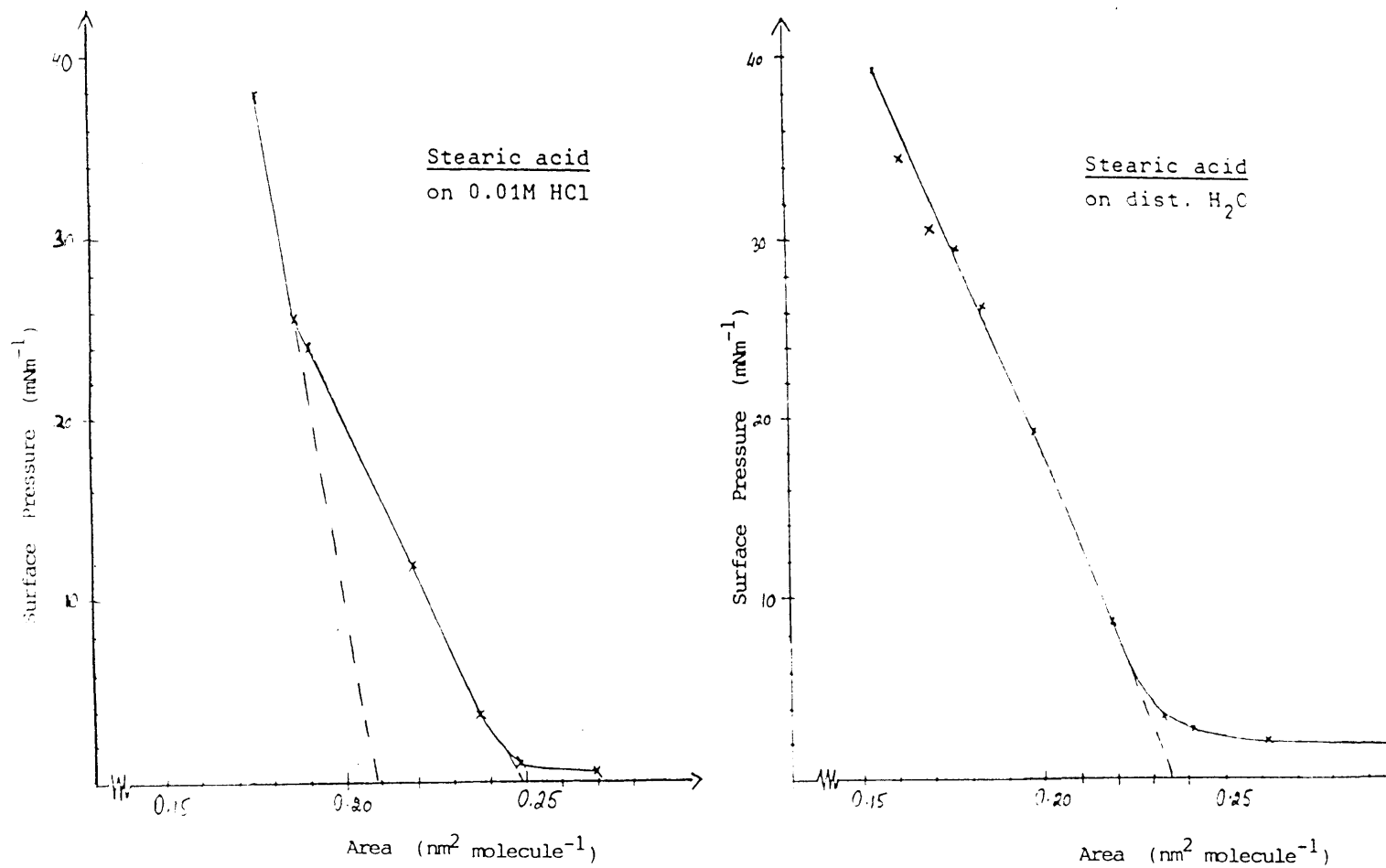


Fig. 3.8 Behaviour of Stearic acid at the air/water and air/acid (0.01M HCL) interfaces

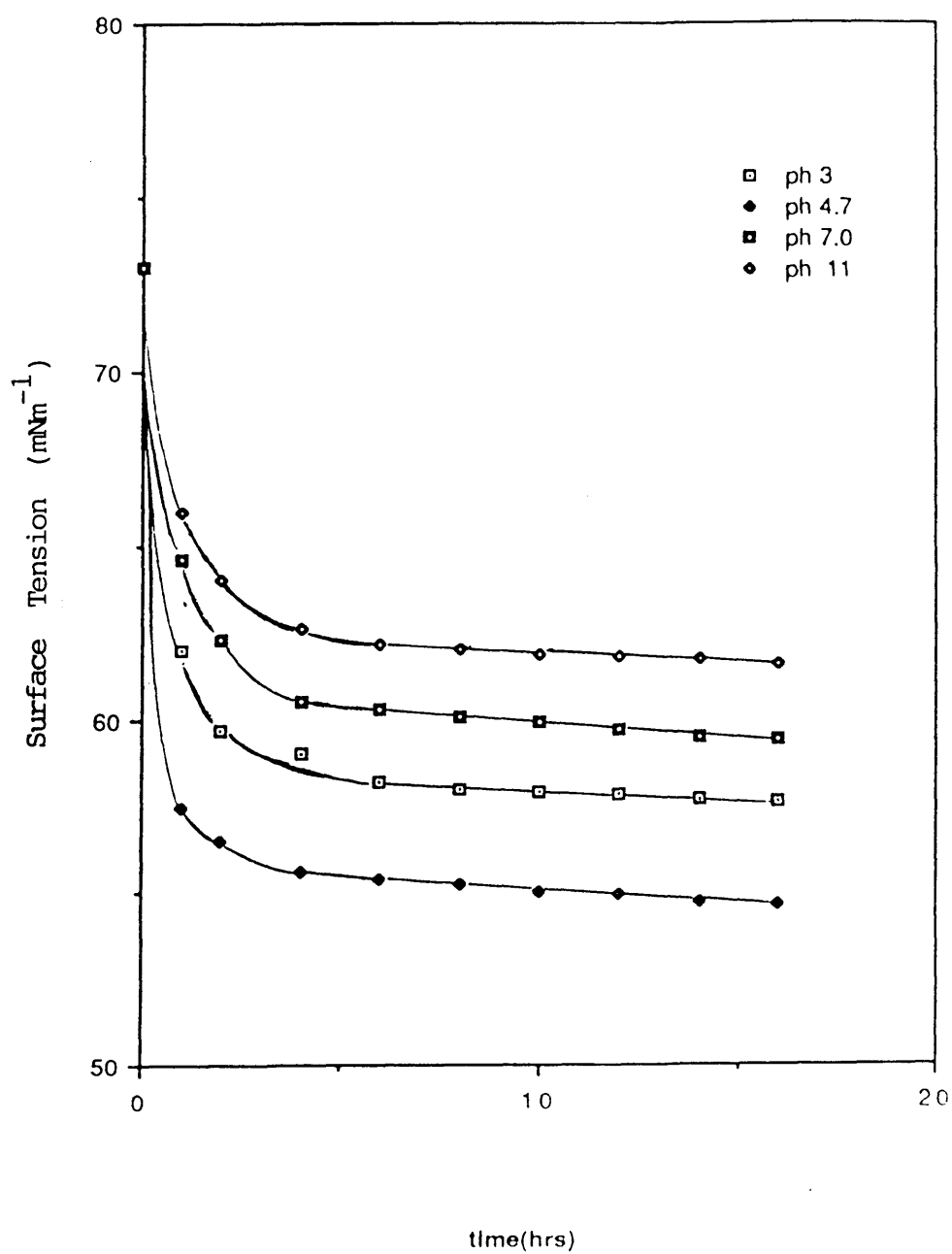


Fig. 3.9 The rate of change in the surface tension values during adsorption of serum albumin is shown for ~ 16 hrs as it reaches equilibrium at an air/water interface. The values for pH 3, 4.7, 7.4 and 11 are given when the bulk concentration is 5×10^{-4} wt%.

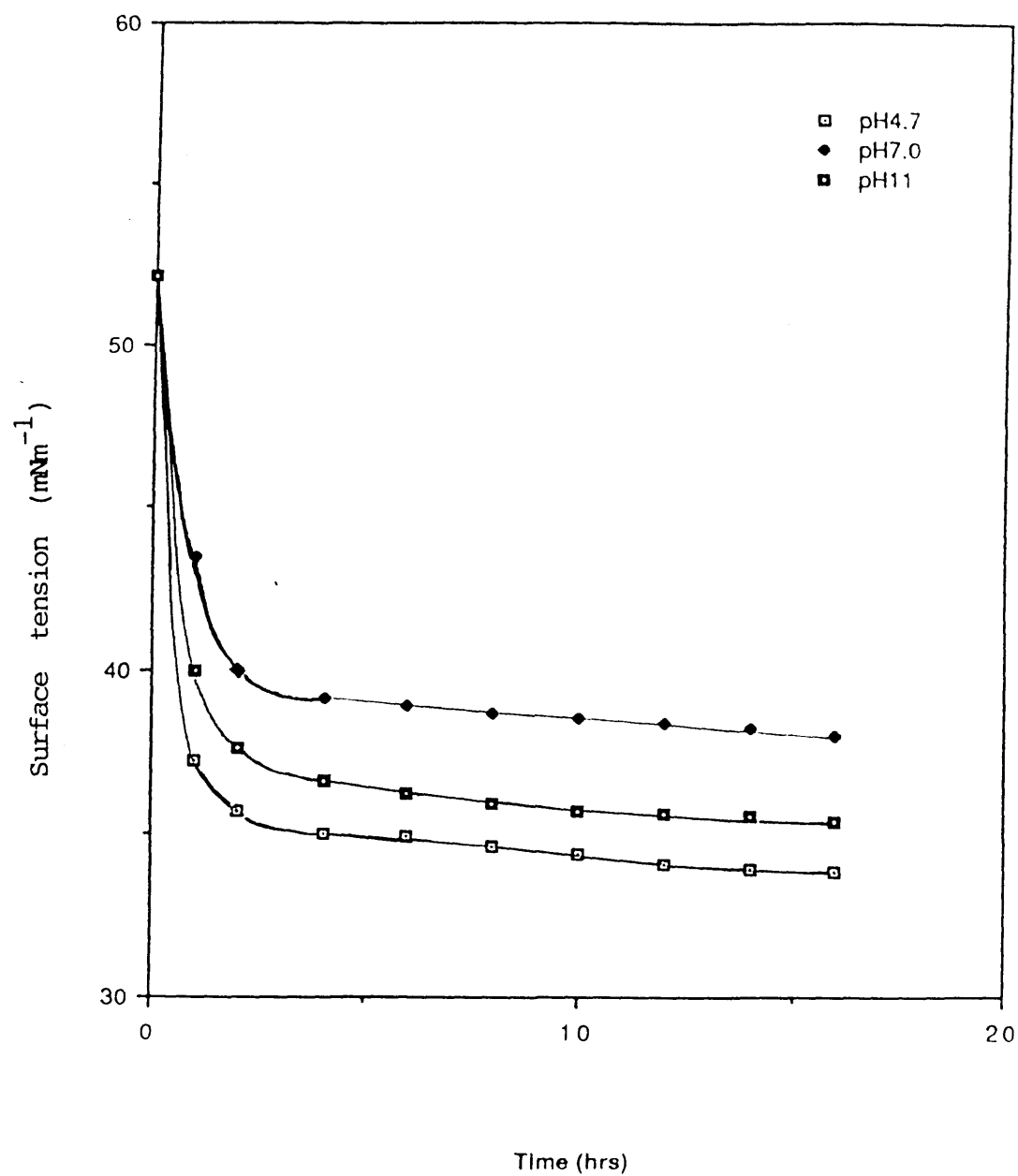


Fig. 3.10 The rate of change in the surface tension values during adsorption of H.S.A. is shown for ~ 16 hrs as it reaches equilibrium at an oil/water interface. The values for pH 4.7, 7.4 and 11 are given when the bulk concentration is 1×10^{-4} wt%.

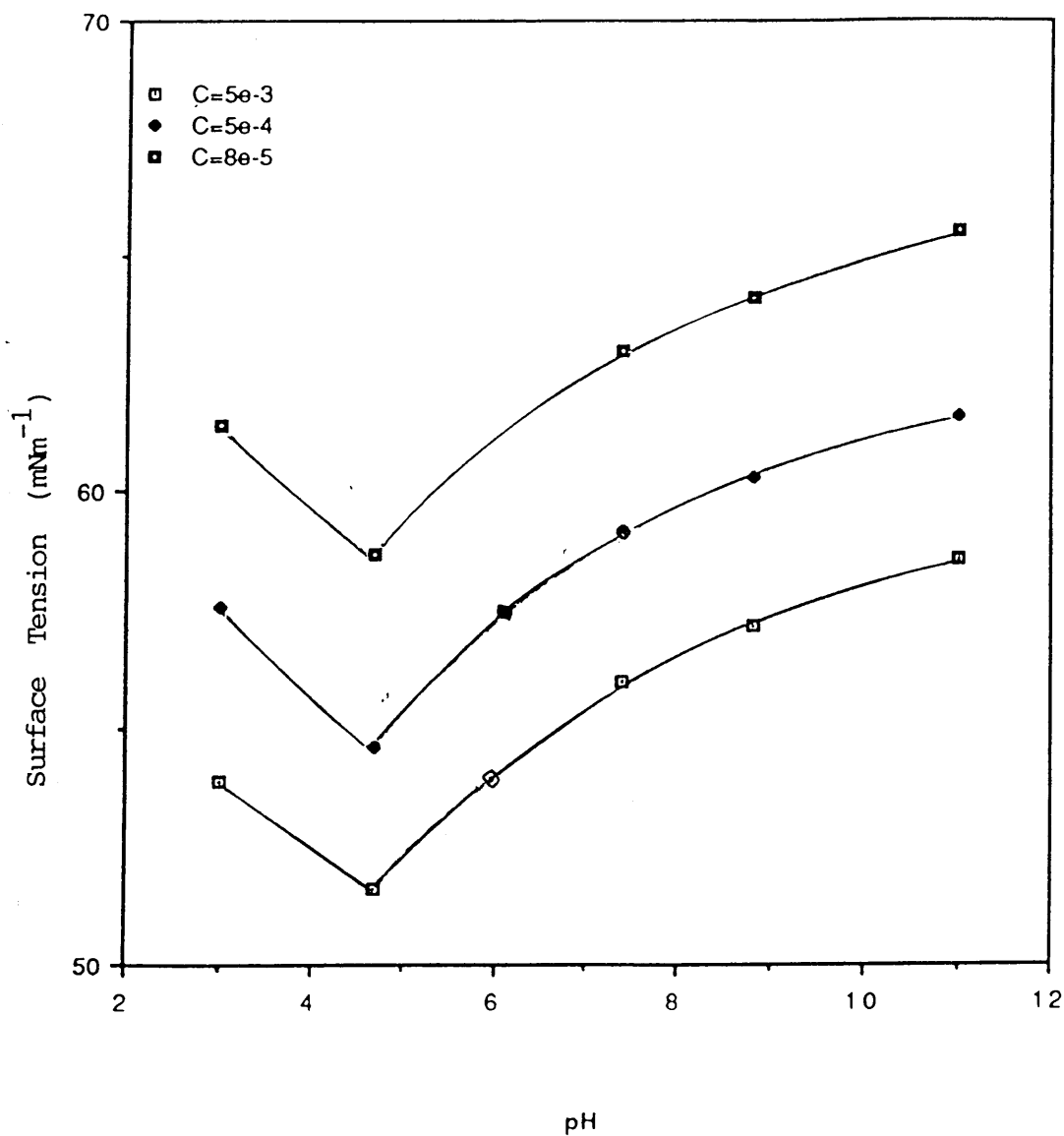


Fig. 3.11 Changes in the surface tension values with variations in pH for different bulk concentration (C_b) at the air/water interface. $C_b = 8 \times 10^{-5}$ wt%, 5×10^{-4} wt% and 5×10^{-3} wt%.

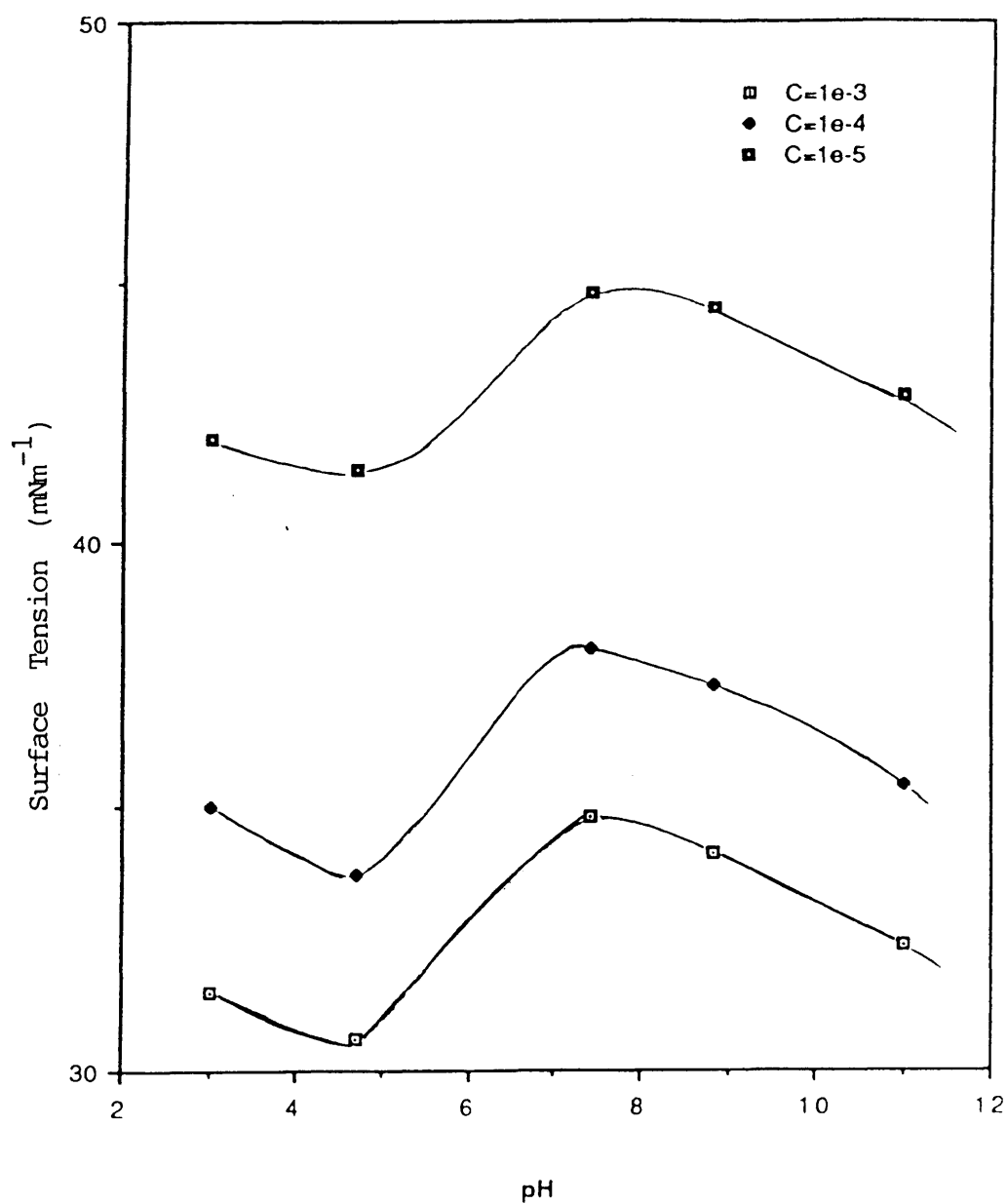


Fig. 3.12 Changes in the surface tension values with variations in pH for different bulk concentrations (C_b) at an oil/water interface. $C_b = 1 \times 10^{-5}$ wt%, 1×10^{-4} wt% and 1×10^{-3} wt%.

3.7.2 Components of surface tension

The intermolecular attractions which give rise to surface tension result from a variety of well-known intermolecular interactions. Fowkes (1964) has shown that the surface tension may be expressed as the sum of components due to the different types of interactions. Thus for liquids 1 and 2 it will be;

$$\gamma_1 = \gamma_1^d + \gamma_1^p \quad 3.6$$

and

$$\gamma_2 = \gamma_2^d + \gamma_2^p$$

where γ^d = surface tension contribution due to dispersion forces, and, γ^p = surface tension contribution due to polar force.

For systems in which only dispersion forces are active, the interfacial free energy is given by

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2 \sqrt{\gamma_1^d \gamma_2^d} \quad 3.7$$

But for systems in which both polar and dispersion interactions occur, the equation has a more general form.

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2 \sqrt{\gamma_1^d \gamma_2^d} - 2 \sqrt{\gamma_1^p \gamma_2^p} \quad 3.8$$

Thus, if the interfacial free energy between an unknown phase and a phase which interacts through dispersion forces only is measured, then the dispersion component of the surface free energy of the unknown

phase can be determined and hence γ^p can be deduced from the surface tension value. If the equilibrium interfacial free energy between a protein solution and a hydrophobic phase of known γ^d is measured then values for γ^d and γ^p for the protein can be determined.

Recently Van Oss *et al.* (1986) considered that it might be of interest to distinguish between long range and short range interactions. Long range interactions were due to London, Debye and Keesom forces and short range interactions mainly involved hydrogen bonding and donor acceptor interactions. Experiments were carried out on protein films adsorbed on solid surfaces. The films were examined in both the hydrated and dried conditions. They used contact angle measurements to measure the components and obtained

$$\Delta G^{TOT} = \Delta G^{LW} + \Delta G^{SR} + \Delta G^{ES} \quad 3.9$$

where ΔG^{TOT} is the total free energy of the interface;

ΔG^{LW} is the sum of the long range forces;

ΔG^{SR} is the sum of the short range forces;

and ΔG^{ES} is the component due to electrostatic interaction.

In their work they assumed that ΔG^{ES} is negligible since the potentials are usually ions and hence write for the interface

$$\gamma_i^{TOT} = \gamma_i^{LW} + \gamma_i^{SR} \quad 3.10$$

In determining γ_i^{LW} they combine liquids with an apolar solid such as PTFE or polystyrene (PST) and with proteins deposited as a thick films on ultrafilter membranes which allow the measurement of γ^d only.

Other parts of the long range interaction, i.e. Keesom & Debye forces, are neglected or appear in the γ^{SR} . Their use of "hydrophobic interactions" is also confusing and usually refers to total interactions of the materials with hydrophobic surfaces rather than the entropic changes which cause the aggregation of apolar materials in water and are partly responsible for the overall conformation of protein molecules in solution. γ^{d} , therefore appears to be the more appropriate parameter to measure.

Since it is likely that the molecules will assume different conformations at different values of the pH, it is reasonable to suppose that values of γ^{d} and γ^{p} will reflect these conformation differences and also differences resulting from the nature of the interface itself. n-decane interacts through dispersion interactions only and has been combined with the protein solutions to determine the interfacial energy and hence γ^{d} and γ^{p} for the protein. The values of γ^{d} and γ^{p} for the pure solvents are given in Table 3.2.

TABLE 3.2

Surface tension components of pure solvents

Solvent	γ (mNm ⁻¹)	γ^{d} (mNm ⁻¹)	γ^{p} (mNm ⁻¹)
Decane	23.83	23.83	0
Buffer	73	21	52

3.8 RELATION BETWEEN STRUCTURE AND COMPONENTS OF SURFACE TENSION

Figs. 3.13 and 3.14 show the components of γ^d and γ^p as a function of bulk concentration. It is seen that both components show changes with concentration. The values of γ^d first increase with increase in bulk concentration reaching a maximum at 10^{-4} wt% after which they decrease. γ^p , however, decreases continuously with increasing bulk concentration. The effect of pH on the conformation of the molecules is shown in Fig. 3.15. γ^d is much greater at pH 11 indicating that the molecule is most hydrophobic due to greater unfolding. The molecule is relatively compact at pH 4.7 and pH 7.3. The values of γ^p show that the polar interactions of HSA are greatest at pH 7.3, the physiological pH.

Changes in the conformation of the molecules in the bulk influence the surface concentration so that the decrease in surface tension is due both to the number of adsorbed molecules and the conformational state they adopt. It is interesting, therefore, to study the changes in γ^d and γ^p with surface concentration Γ .

Surface concentration is determined directly by the radio tracer method which is explained in detail in Chapter Four. Figs. 3.16 and 3.17 show components of surface tension plotted as a function of surface concentration.

These graphs may be compared with Figs. 3.13 and 3.14. The curves show the same general trend. However, at a low value of the surface concentration, Γ , the contribution due to γ^d is seen to be lowest at pH 4.7, and highest at pH 11.

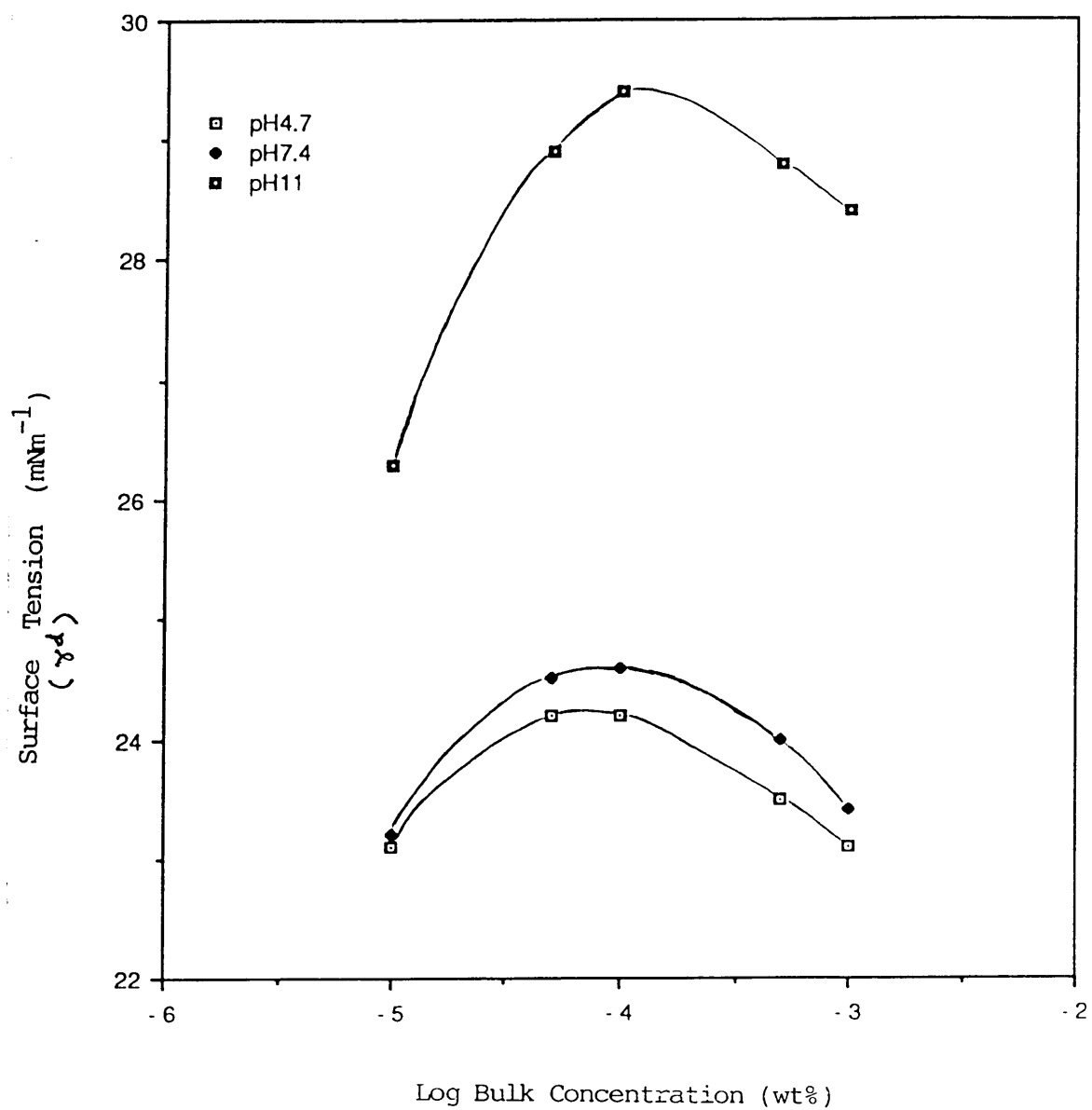


Fig. 3.13 Contribution of the dispersion forces to the surface tension values as the bulk concentration increases for different pH values.

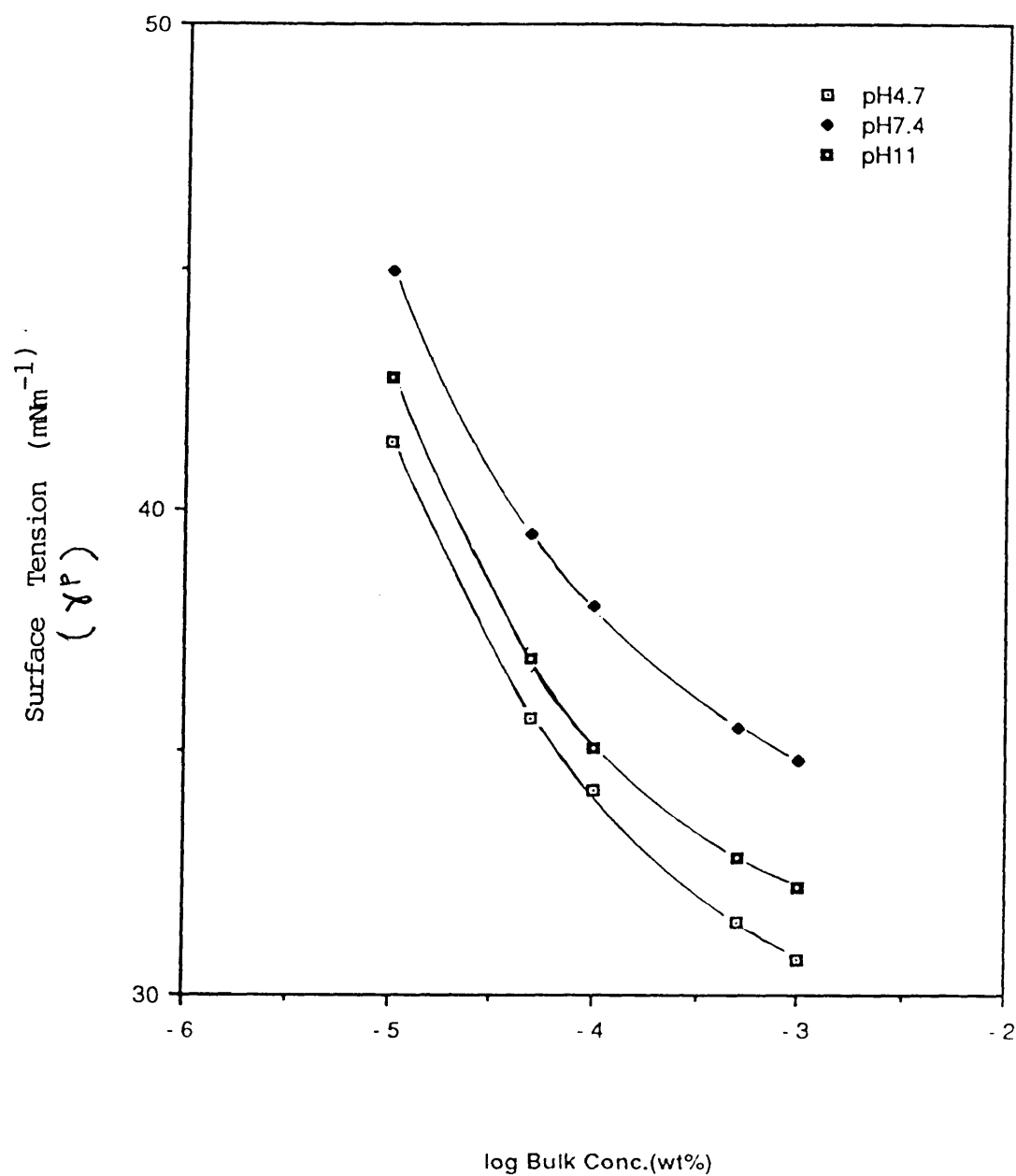


Fig. 3.14 Contribution of the polar forces to the surface tension values as the bulk concentration increases for different pH values.

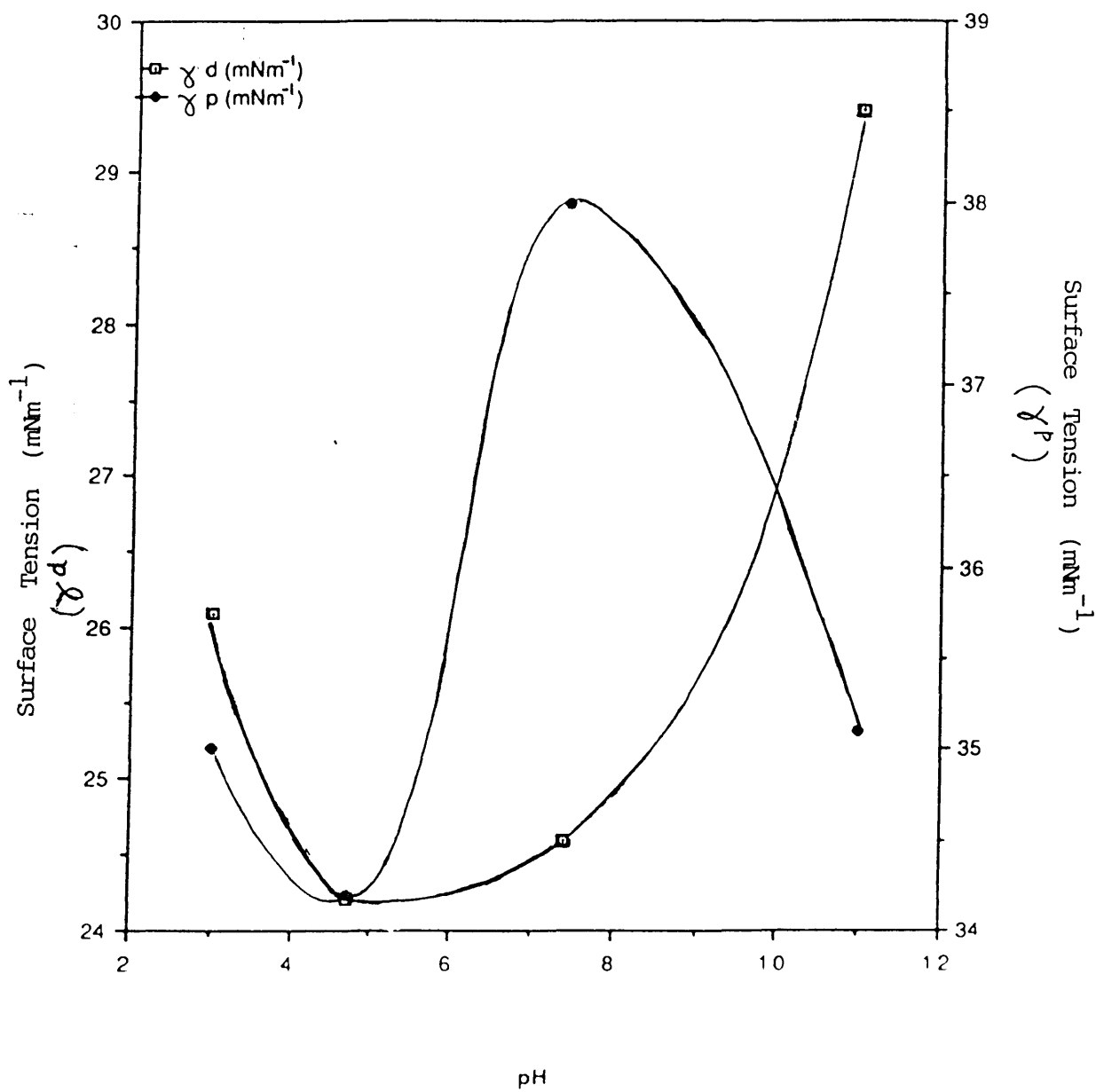


Fig. 3.15 The changes in the dispersion and polar forces as the pH of the solution is changed for a particular bulk concentration of 7.7×10^{-5} wt %.

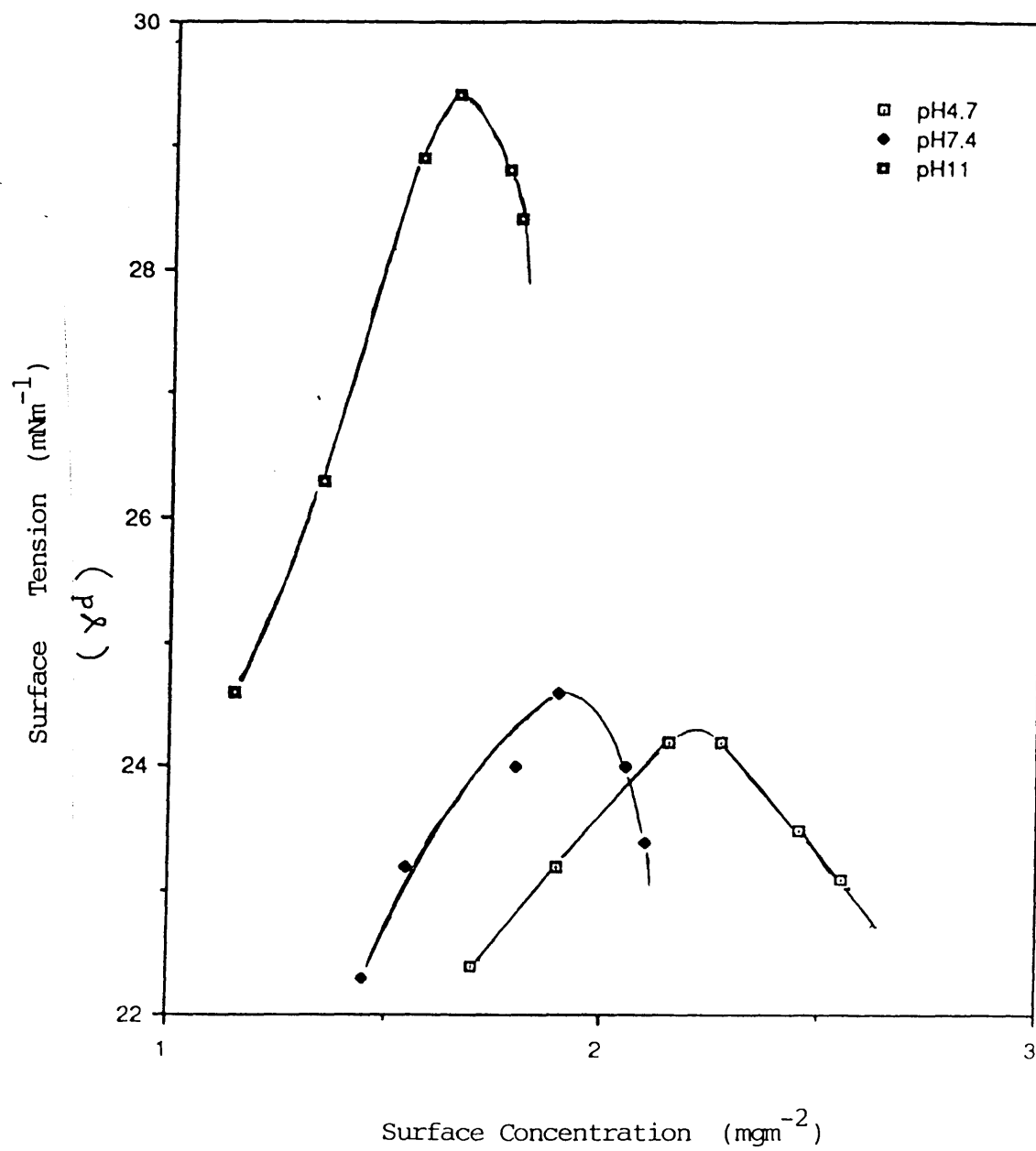


Fig. 3.16 Changes in dispersion forces as surface concentration is varied for pH4.7, pH7.4 and pH11.

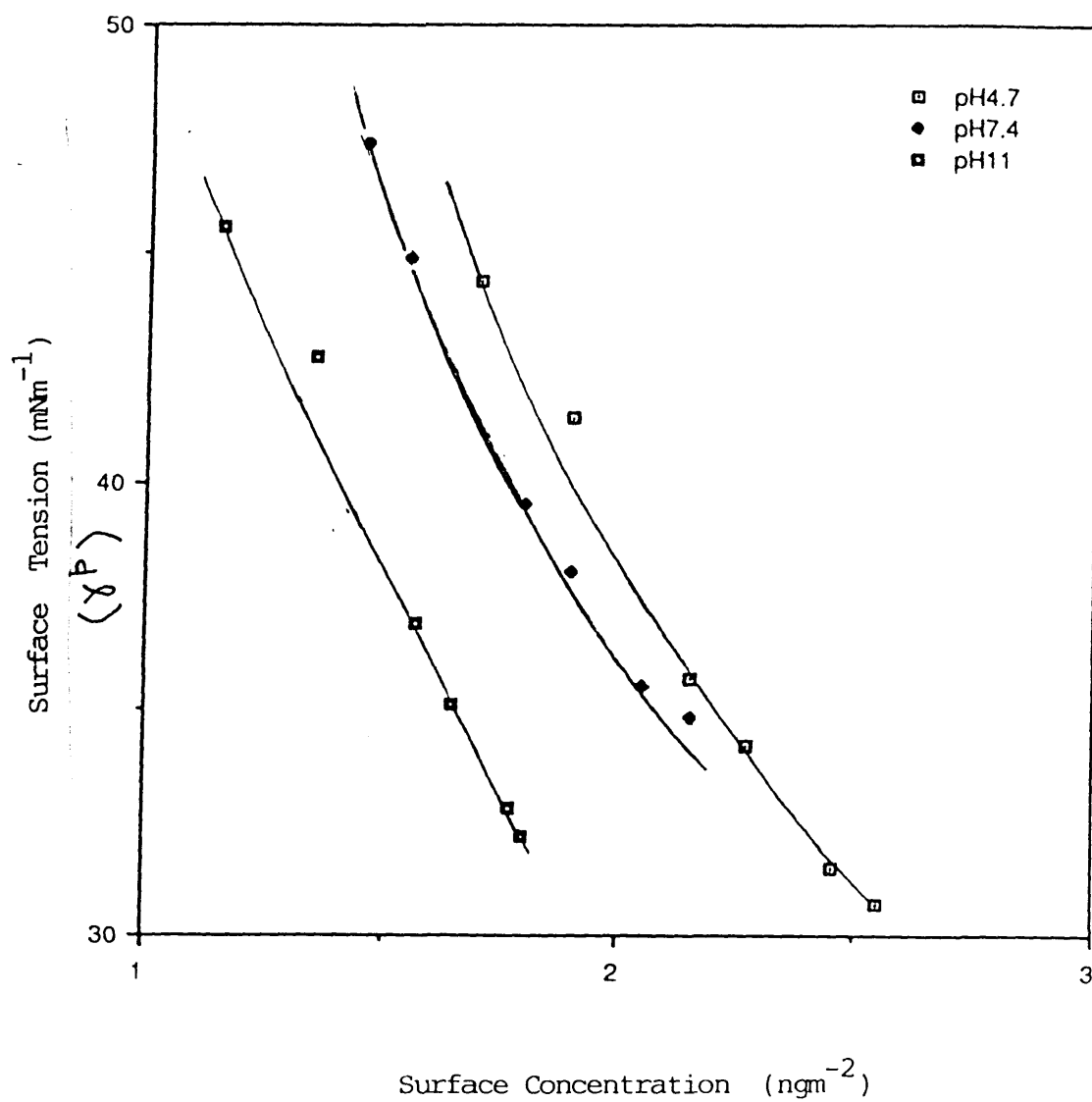


Fig. 3.17 Changes in the values of the polar forces as the surface concentration is increased for pH4.7, pH7.4 and pH11.

$1/\Gamma$ represent the area of surface available to a molecule. The area of the protein molecules themselves is different in the two cases because of conformational changes. If R is the radius of a circle having the same area as the molecule assumes on the surface, then $\Gamma\pi R^2$ is the ^{fractional} extent of the interface which is covered with protein molecules. R is very much smaller at pH 4.7 than at pH 11 as indicated in Fig. 3.18.

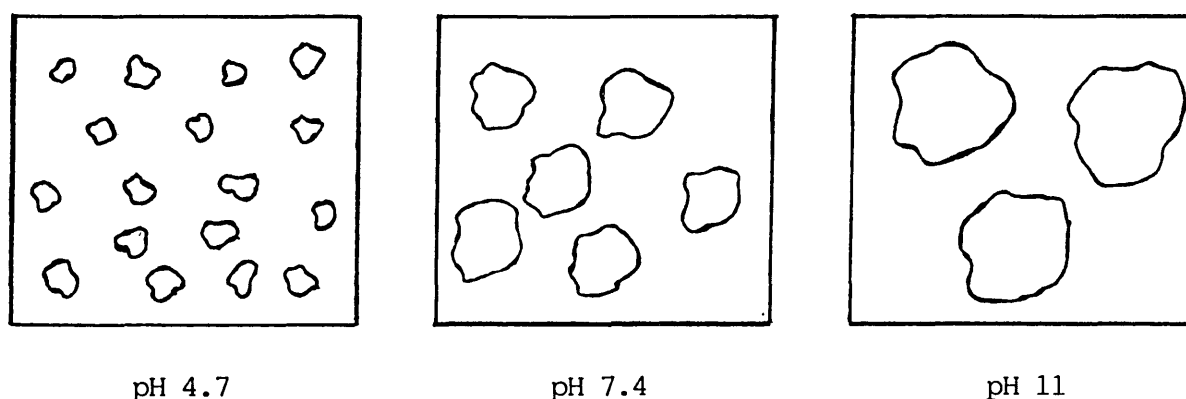


Fig. 3.18 The extent of interface covered with protein molecules for different pH values.

Hence the contribution to γ^d per molecule is least at pH 4.7 and rises as the pH increases as shown in Fig. 3.19. The polar contribution, γ^p , is to a large extent due to the uncovered water surface. When γ^p is plotted against bulk concentration (Fig. 3.15), the highest values are obtained for pH 7.4. The reason for this is that the molecule is most soluble at pH 7.4.

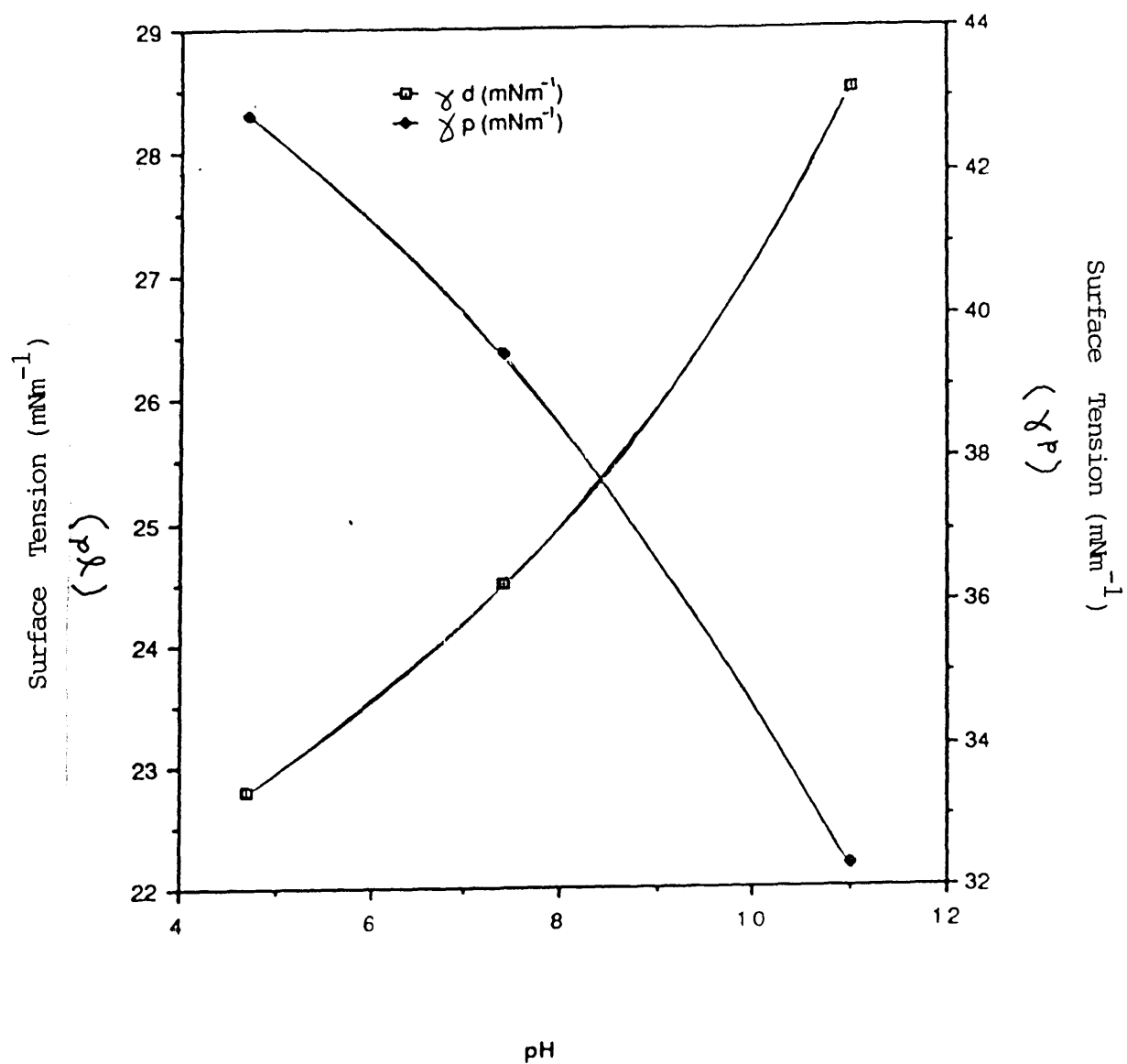


Fig. 3.19 Changes in the dispersion and polar forces as the pH of the solution is changed for surface concentration = 1.8 mgm^{-2} .

Hydrogen bonds form the major components of polar interactions. In proteins many possibilities for hydrogen bonding are available. Among these, the hydrogen bonds between the peptide units and H_2O are the most numerous, viz. 60-80%, the fraction of hydrogen bonds between a peptide unit and a side chain and that between side chains generally are in the range of 15-30% and 5-15% respectively (Privalov, 1974).

From the model studies of peptide hydrogen bond formation Krescheck & Klotz (1969) concluded that in the apolar phase hydrogen bonding between peptide units is favourable whereas in aqueous media, it is not. Moreover, Tanford (1970) showed that disruption of peptide-peptide hydrogen bonds in the interior of the molecule, followed by exposure of these peptide units to the aqueous medium, is a thermodynamically favourable process. This shows that peptide hydrogen bonding does not contribute to the formation of a compact protein structure. However, from helix-coil transitions of synthetic polyamino acids Norde *et al.* (1979) inferred that intramolecular peptide hydrogen bonds are more favourable than peptide-water interactions. It is obvious that variations in hydrogen bonding is one of the factors which affect the rotational freedom of the polypeptide chains.

3.9 DETERMINATION OF AVERAGE MOLECULAR SIZE

Since gel filtration is a reliable separation technique, it was employed here to study the changes in the molecular size of HSA for different values of pH.

The basic principle of column chromatography is that as a solute passes down a chromatographic bed its movement depends upon the bulk flow of the mobile phase and upon the Brownian motion of the solute

molecules which causes their diffusion both into and out of the stationary phase. The separation in gel filtration depends on the different abilities of the various molecules to enter pores of the stationary phase. Very large molecules which never enter the stationary phase move through the chromatographic bed faster than the smaller molecules, which can enter the gel pores and therefore move more slowly through the column.

The reliability of gel filtration stems from the stability of Sephadex and other gel filtration media and their inertness towards biopolymers under a wide range of conditions.

Sephadex Type G which is an accepted standard for gel filtration is available in different particle size grades. Here the superfine grade G-200 which is intended for column chromatography requiring a higher resolution was used. It can be used for the filtration of molecules up to a molecular weight of about 600,000.

Unless chemically degraded, Sephadex is insoluble in all solvents. It is stable in water, salt solution, organic solvents, alkaline and weakly acidic solutions. It does not melt and can be sterilized in the wet state at neutral pH or by autoclaving for 30 minutes at 120°C without affecting its chromatographic properties. For good resolution, the equipment for the column should also be chosen carefully.

Gel filtration results are expressed in the form of an elution diagram which shows the variation of solute concentration in the eluent with the volume of the eluent passed through the column. A permanent record can be obtained if it is continuously detected using a u.v. monitor and a recorder.

The elution volume (V_e) varies with the total volume (V_t) that is passed through the column. The elution volume of the molecules which are only distributed in the mobile phase as they are larger than the pores of the gel is the void volume (V_o). They are measured by the elution volume of the radioactive ions. Here I^{125} labelled albumin was used.

The fraction of the stationary gel volume (κ_{av}) which is available for diffusion of a given solute species is represented by

$$\kappa_{av} = \frac{V_e - V_o}{V_t - V_o} \quad 3.11$$

In gel filtration, no molecules can be eluted with a κ_{av} greater than 1 or less than 0. If the κ_{av} is less than 0, channeling in the chromatographic bed is indicated and the column must be repacked.

Results of HSA passed through Sephadex G-200 at pH 3.4, 7.0 and 11 are shown in Figs. 3.20, 3.21 and 3.22. Molecules of different radii are eluted as though they had different values of molecular weight which can be deduced from κ_{av} using a calibration curve. The values for HSA are given in Table 3.3.

TABLE 3.3

Molecular weight deduced from κ_{av} using a calibration curve obtained with a series of spherical molecules

pH	κ_{av}	Molecular weight
3.4	.39	92 000
4.7	.47	65 000
7.4	.43	80 000
11	.32	150 000

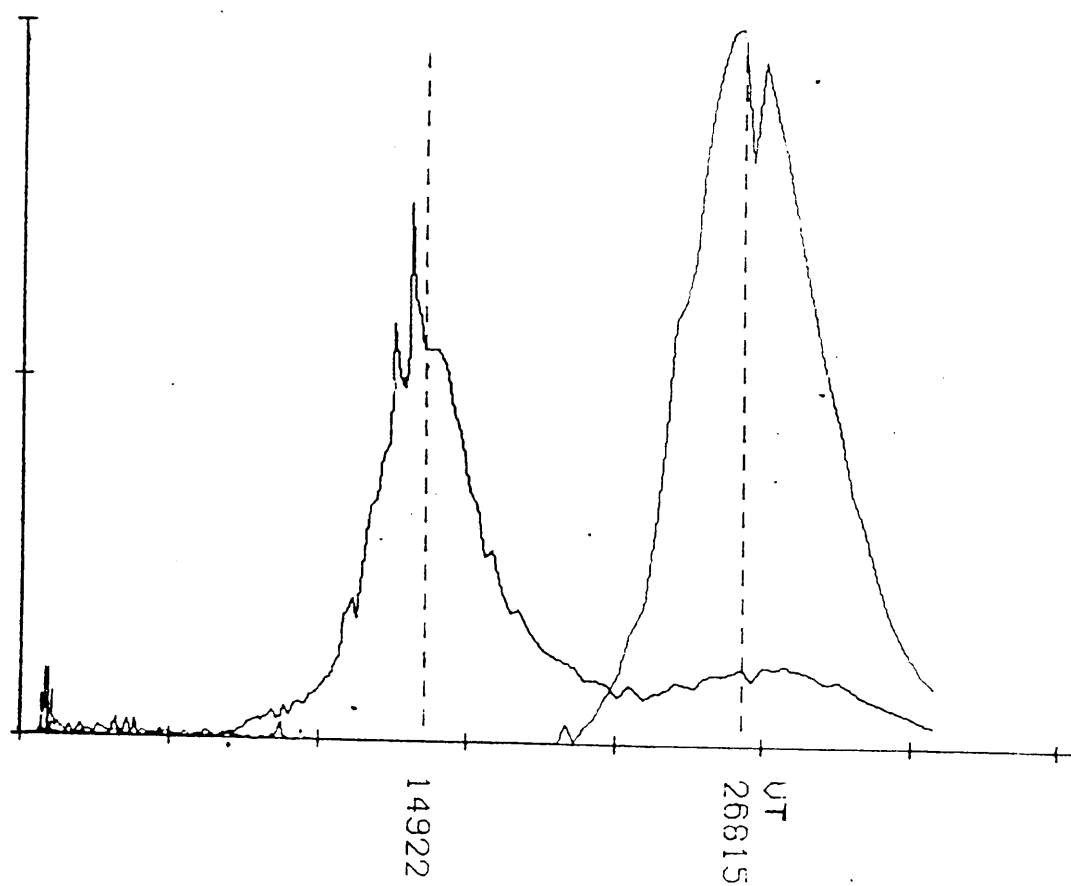


Fig. 3.20 Results of HSA passed through Sephadex column at pH3.4.

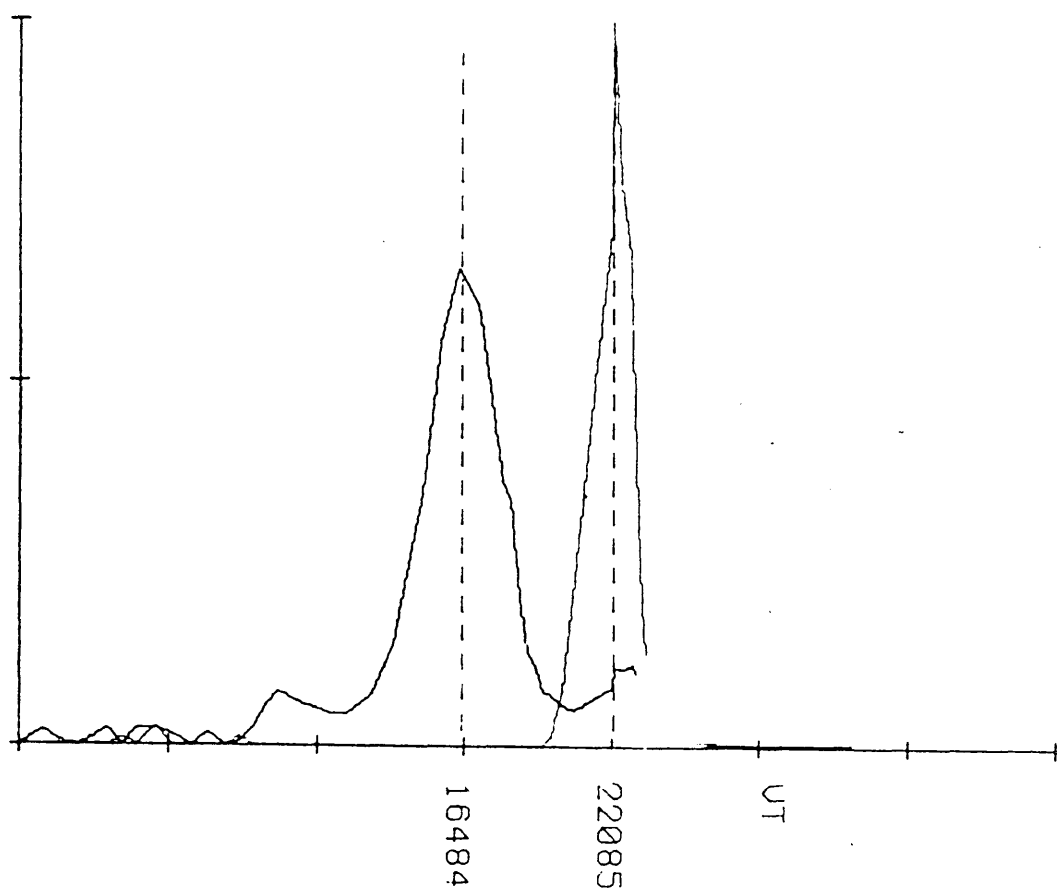


Fig. 3.21 Results of HSA passed through Sephadex column at pH7.

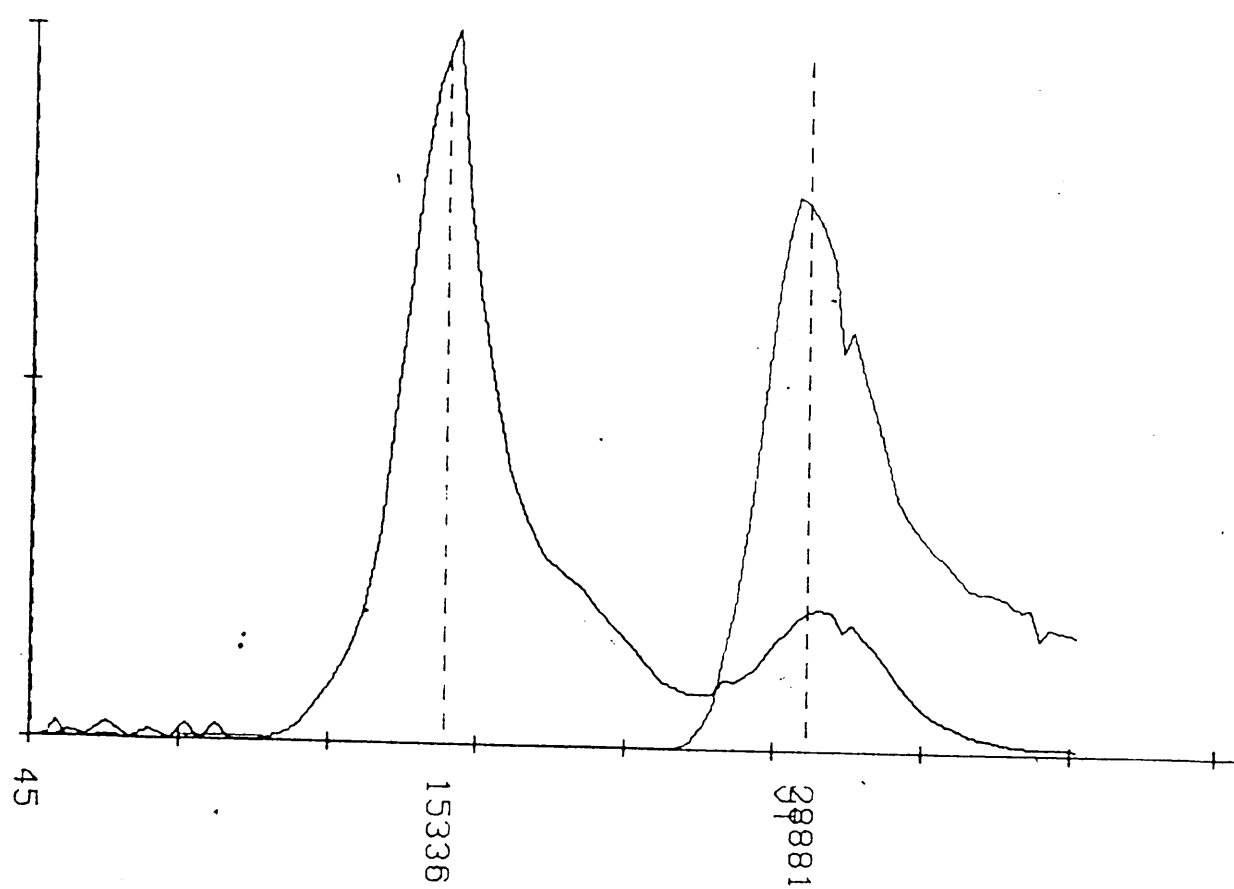


Fig. 3.22 Results of HSA passed through Sephadex column at pH11.

3.10 EXISTENCE OF PARTIALLY UNFOLDED MOLECULES IN THE ADSORBED LAYER

Many concepts have been put forward to explain the structure of a molecule at an interface. The work of Lyman & Kim (1971), Brash & Lyman (1969) suggests that the molecule adsorbs in an intact globular form with minimal configurational change but Scheller *et al.* (1975) from work on adsorption of albumin on mercury conclude that within 30 sec. of exposure a completely unfolded monolayer is obtained. Step compression of an equilibrated film have been carried out on the surface of water. A sample of the results is shown in in Fig. 3.23. It is seen that after a sudden decrease, the surface tension relaxes but does not reach the equilibrium value. This shows that some of the molecules have been desorbed during compression suggesting that the concept of identical close packed, irreversibly adsorbed protein molecules is not valid. However, it does suggest that there is a component of the interfacial film which has limited compressibility and differs from the intact globular form and is irreversibly adsorbed. Before compression the adsorbed film will consist of molecules in a range of conformations, those which arrived early expand the most while late arrivals adopt a nearly native configuration (see Fig. 3.24). On compression it is these late arrivals which are able to return to the bulk phase leaving a surface which has a lower intrinsic surface tension than the original surface.

Additional observations which support this hypothesis are shown in Fig. 3.25. In this case the adsorbed film of HSA was subjected to continuous expansion and compression. The surface tension rises during expansion, more molecules adsorb many in the native state. In the compression part of the cycle some of these leave the surface and

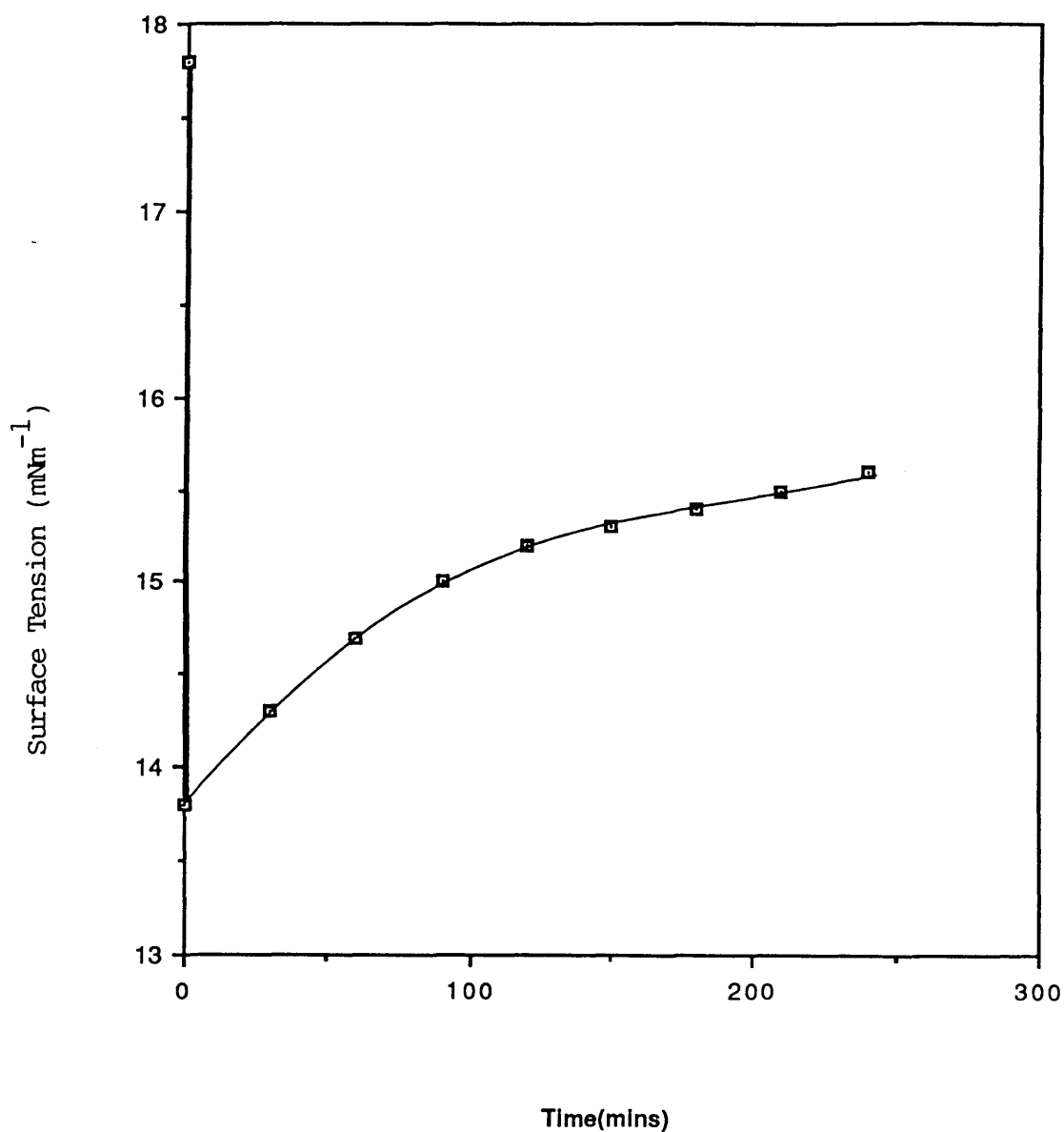


Fig. 3.23 Changes in the surface tension values after step compression of an equilibrated film of protein at pH7.4. Initial drop occurs from 17.8 mNm⁻¹ to 13.8mNm⁻¹. The surface tension values gradually increase but do not reach the equilibrium values.

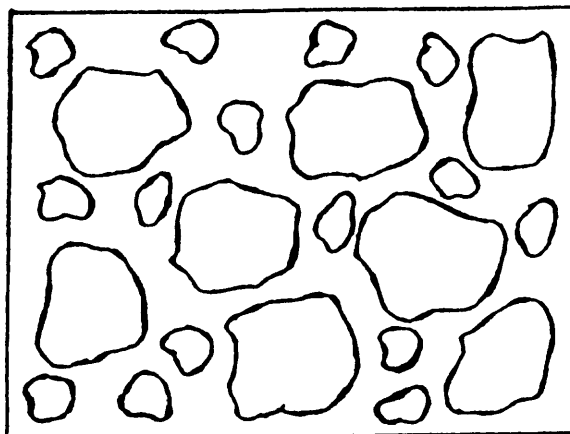


Fig. 3.24a Different configuration the molecule adopts upon adsorption.

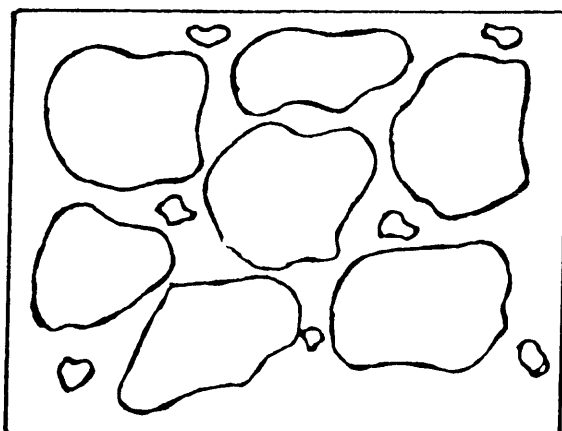


Fig. 3.24b On compression the molecules in their native state get desorbed.

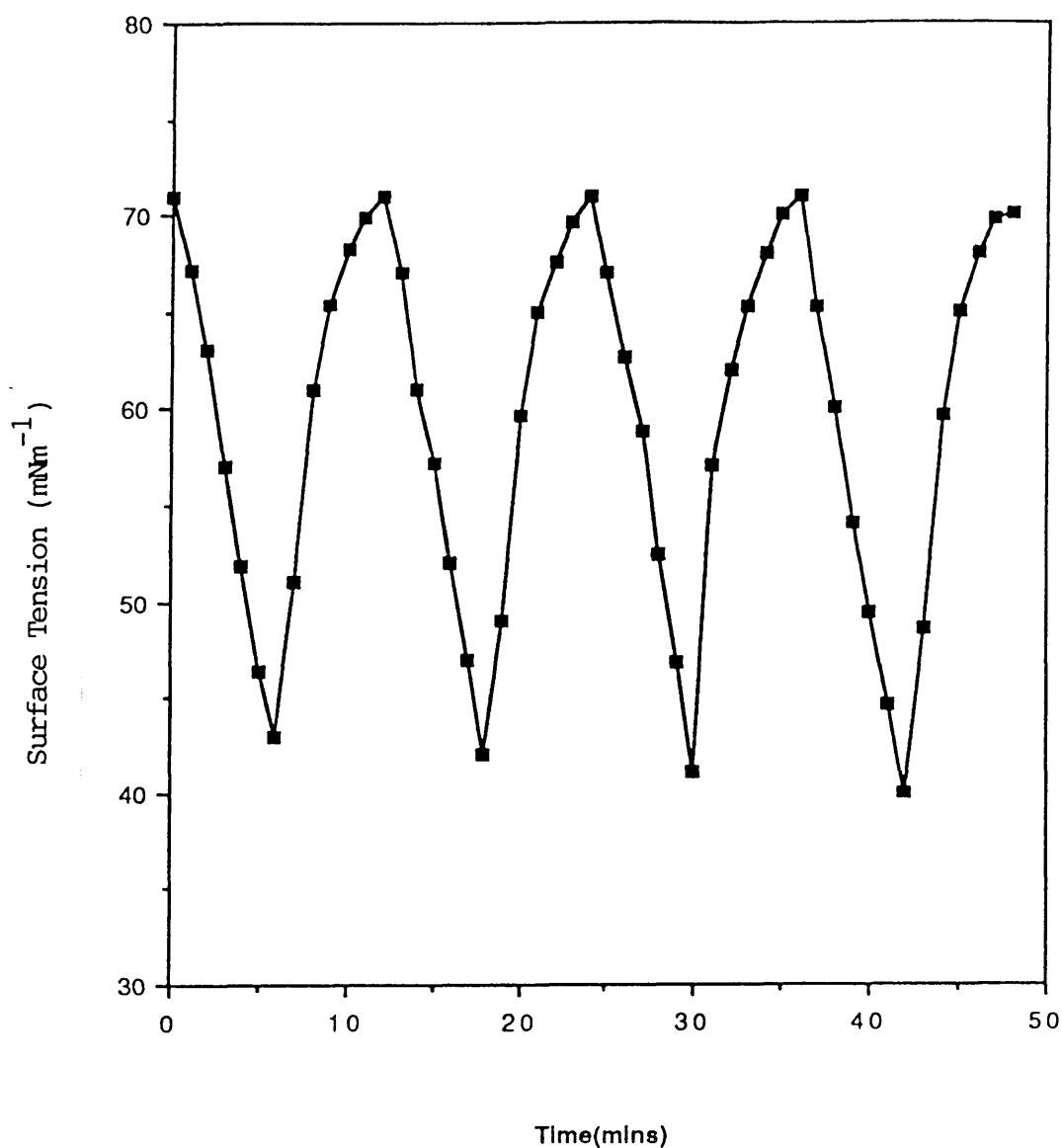


Fig. 3.25 Changes in the surface tension during continuous expansion and compression. On expansion the molecules adsorb many molecules in their native state, some of which are desorbed as the surface is compressed. On further expansion the native molecules still in the adsorbed film unfold and more adsorption also occurs. The concentration of the unfolded irreversibly adsorbed molecules therefore increases. This becomes clear as surface tension attains a lower value during successive compression.

the surface tension decreases. During the second expansion the native molecules still in the adsorbed film unfold and more adsorption occurs. The concentration of unfolded, irreversibly adsorbed, molecules has therefore increased. This is evident from the fact that the surface tension attains a lower value after the second compression. The process continues with subsequent expansions and compressions.

The work shows that although surface tension is a relatively simple measurement it is sensitive both to adsorption and to conformational changes which occur in the adsorbed film.

CHAPTER FOUR

THE DETERMINATION OF INTERFACIAL CONCENTRATION OF PROTEINS

4.1 INTRODUCTION

The equilibrium interfacial concentration of protein depends not only upon the rate of adsorption but also on the conformation which the molecule adopts after adsorption. The conformation depends on the relative magnitude of the interchain attraction compared with the attraction of the chain segments for the substrate.

These interactions depend upon many factors such as surface concentration, molecular weight of the protein, the degree of flexibility of the chains, the charge carried by the molecule and also on the properties and purity of the liquids which form the interface.

The study of monolayer interactions at the surface has been pursued vigorously since 1765 when Benjamin Franklin first performed the experiments of spreading oil on water and found that the thickness of the spread film was never less than $25\text{\AA}$. The parameter which describes the structure of the molecules at an interface is the limiting area which they assume. Therefore the equilibrium surface pressure, π , vs. molar area, A , relationship has invariably been used for assessing interactions in monolayers. Although the theoretical interpretation of $\pi:A$ curves and even the nature of surface interactions may be controversial they are known to influence both the static and dynamic surface properties.

The surface pressure of a protein film is complicated in that it depends not only on the surface concentration but on the conformation of the molecules as well. In addition, the method by which the film

is produced is also important as adsorbed films are essentially different from those obtained by spreading the material from a solvent.

4.2 THE MEASUREMENT OF SURFACE CONCENTRATION

4.2.1 Direct measurement of surface concentration

As knowledge of interfacial concentrations, Γ or molar area ($A = 1/\Gamma$), is essential in gaining an understanding of the behaviour of a monolayer, several methods, direct or indirect, have been used to measure it.

Techniques which have been used for direct measurements at an air/water interface include:

4.2.1.1 The microtome method: This was used by McBain (1936, 1940a). Here a blade skims off a depth of $\sim 50\mu\text{m}$ from the surface of liquid in a large trough. The surface excess is calculated as a difference between the concentration of this surface layer and that of the bulk. The advantage of this system is that as the blade moves rapidly the equilibrium is not disturbed. However, the problems of alignment render this technique very difficult to use.

4.2.1.2 Surface compression method: Another simple method used by McBain (1940(b)) involved rapid compression of the surface of a liquid held in a special trough. Desorption of the surplus material from the surface increases the bulk concentration which is then determined interferometrically. This technique also required exceptional accuracy resulting in limitations in its use.

4.2.1.3 Ellipsometric technique: This has also been used to determine the amount of protein adsorbed. Smith *et al.* (1970) used this technique to measure the amount of protein adsorption on metal and polyethelene surfaces. The method is based on the phenomenon that the ellipticity of a plane-polarized beam of light is changed on reflection by a thin film at the interface. Generally this technique has been used to determine the surface concentration and the thickness of films adsorbed on solids but Benjamin *et al.* (1975) have extended this technique to water soluble proteins at air/liquid interfaces.

4.2.1.4 Infra red spectroscopy: This technique was used by Kim & Lee (1974) in their search for a biocompatible material. They studied the adsorption of proteins on different polymers and showed that it was strongly substrate dependent. This technique has been limited in its application to proteins on solids mainly due to the problem of working with aqueous solutions. Deuterium oxide (D_2O) is often substituted for water to allow for observation of the amide I and II bands of the proteins without interference from the OH-bonding of the water molecule. However, the hydrogen deuterium exchange which occurs rapidly when proteins are placed in D_2O causes spectroscopic changes and perhaps conformation changes as well. It is also known that quantitative studies of proteins adsorbed at the surface is more difficult than for protein solutions. However, Fink *et al.* (1984) have improved the technique by using radio labelled proteins in conjunction with FT-IR to calibrate the spectroscopic

technique so that absolute quantity of adsorbed protein may be obtained.

4.2.1.5 Electron microscopy: Gorman *et al.* (1971) studied the adsorption of proteins on mica and evaporated carbon by using electron microscopy. They visualized and directly counted the number of molecules adsorbed. However with an increase in concentration, it became difficult because of overlapping, to distinguish and count the individual molecules.

4.2.1.6 Total internal reflection fluorescence (TIRF): TIRF is an optical technique used to examine the interaction of macromolecules with solid surfaces. It was used by Lok *et al.* (1983) to obtain both the adsorption rate and equilibrium data for fluorescently labelled proteins. They found that at low concentration the contribution of the bulk solution to the total TIRF signal was negligible and at the higher bulk concentrations the TIRF adsorption record was more difficult to interpret because bulk fluorescence contributed significantly to the total fluorescence signal and thus complicated the experimental results. Therefore it was essential that all contributions due to the bulk solution must either be eliminated or accurately quantified so that signals arising from surface reaction only were obtained.

4.2.1.7 Counting of radio-active labelled proteins: This has been one of the most successful methods employed in the study of adsorption of proteins both at the liquid and the solid inter

faces. Several other methods of determining surface concentration were considered and tried out. The most reliable was the radio-tracer technique and this was adopted for all the work quoted here. In fact, according to Muramatsu (1978) if the experimental conditions of this technique are adequately arranged so that the proportionality between the mass of the labelled substance and the activity is known, then the data obtained by this method is as reliable as the mass being weighed by a balance.

A measure of the surface radioactivity is obtained using a counter placed directly above the interface. It detects radiation from molecules close to the interface. If soft β particles are used, then the emission has a small range. This avoids large errors due to radiation from the bulk hence giving a direct measurement of surface concentrations.

4.2.2 Indirect determination of surface concentration

4.2.2.1 Gibbs adsorption equation: The exact mathematical treatment for determining the excess of the solute in the surface or interface was first made by Gibbs (1931). His equation is regarded as one of the most fundamental thermodynamic treatments of the interfacial phase. It enables the extent of adsorption at a liquid surface to be estimated from the surface tension data.

For the quantitative treatment of the surface phenomenon it is convenient to regard the interface between two phases as a mathematical plane such as S-S as shown in Fig. 4.1.

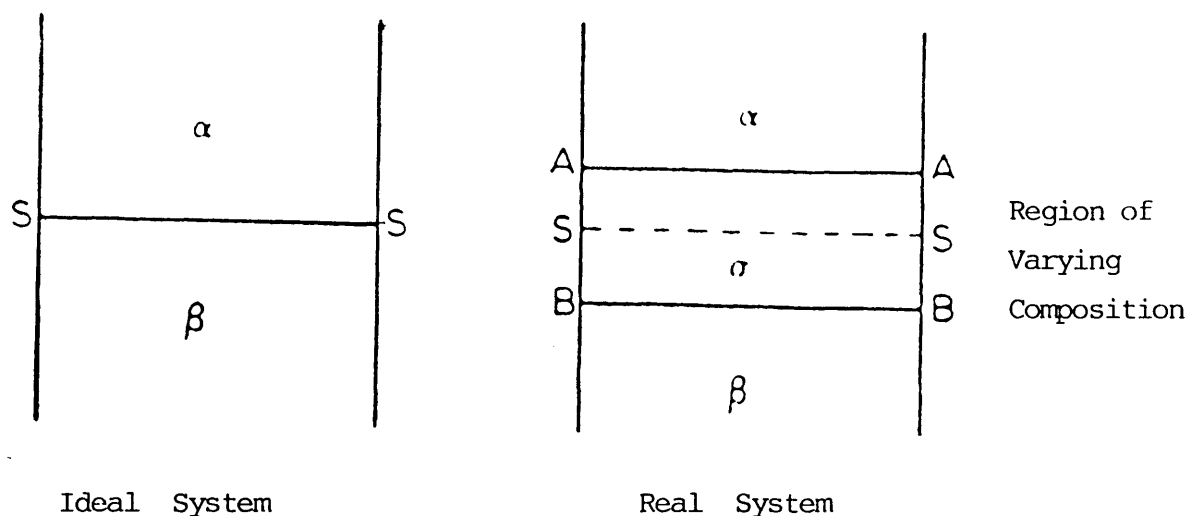


Fig. 4.1 Representations of an interface between bulk phases α and β

Adsorption is then conveniently expressed in terms of surface excess concentrations of component 1 as

$$\Gamma_1 = \frac{\eta_1^\sigma}{A} \quad 4.1$$

where η_1^σ is the amount of component 1 in the surface phase σ , and A is the interfacial area.

The total-thermodynamic energy of the surface phase σ is given by

$$U^\sigma = TS^\sigma - PV^\sigma + \gamma A + \sum u_i \eta_i^\sigma \quad 4.2$$

where U = total internal energy, S = entropy, P = pressure, V = volume, and u = the chemical potential.

PV^σ and γA have opposite signs since pressure is an expanding force and surface tension is a contracting force. By differentiating Eq. 4.2, we get

$$dU^\sigma = TdS^\sigma + S^\sigma dT - PdV^\sigma - V^\sigma dP + \gamma dA + Ad\gamma + \sum u_i d\eta_i^\sigma + \sum \eta_i^\sigma du_i \quad 4.3$$

From the first and second law of thermodynamics for a surface phase we have

$$dU^\sigma = TdS^\sigma - PdV^\sigma + \gamma dA + \sum u_i d\eta_i^\sigma \quad 4.4$$

Subtracting Eq. 4.4 from Eq. 4.3, we get

$$S^\sigma dT - V^\sigma dP + Ad\gamma + \sum \eta_i^\sigma du_i = 0 \quad 4.5$$

Therefore at constant temperature and pressure

$$d\gamma = - \sum \frac{\eta_i^\sigma}{A} du_i = - \sum \Gamma_i du_i \quad 4.6$$

For a simple two-component solution, i.e. consisting of a solvent and a single solute, Eqn. 4.6 becomes

$$d\gamma = - \Gamma_A du_A - \Gamma_B du_B$$

If the surface excess concentration of the solvent (Γ_A) is zero. The above expression simplifies to

$$d\gamma = - \Gamma_B du_B$$

Chemical potential changes can be related to relative activities by

$$u_B = u_B^\ominus + RT \ln a_B \quad 4.7$$

$$\text{then} \quad du_B = RT d \ln a_B \quad 4.8$$

where $R = G$ as constant,

$T =$ absolute temperature,

and $a =$ relative activity.

Therefore

$$\Gamma_B = -\frac{1}{RT} \cdot \frac{d\gamma}{d \ln a_B} = -\frac{a_B}{RT} \cdot \frac{d\gamma}{da_B} \quad 4.9$$

or for dilute solutions, when the interfacial layer is in equilibrium with the bulk and assuming that at low concentrations the activity coefficient is unity the following equation is obtained

$$\Gamma_B = -\frac{C_B}{RT} \cdot \frac{d\gamma}{dC_B} \quad 4.10$$

Hence, from the slope of the surface pressure isotherm, the values for surface concentration can be calculated. The Gibbs adsorption isotherm covers a wide field as it applies to all solutions which are dilute and the solute present on them are dispersed in simple form and not as molecular aggregates like soap micelles.

Gibbs adsorption equation was used to verify the adsorption isotherm obtained by the radio tracer method as previous workers have reported excellent agreement of values for surface excess obtained in

this way. However, these theoretical values are found to be in good agreement with experimental values at low concentrations only.

4.2.2.2 The determination of surface concentration from dynamic surface tension measurements: Adsorption isotherms for soluble monolayers can also be determined by dynamic surface tension measurements. When a surface is expanded the surface tension changes and we can define the dilatational modulus ϵ as

$$\epsilon = \frac{A d\gamma}{dA} \quad 4.11$$

The modulus is very sensitive to interactions between the adsorbed molecules and is also dependent on the rate of change of the area. It may also be expressed as

$$\epsilon = - \frac{d\pi}{d \ln A} \quad 4.12$$

where π is the surface pressure ($\pi = \gamma_0 - \gamma$).

The dilatational properties of monolayers yield interesting insights into the relaxation processes occurring with time. These will be discussed in detail in Chapter Five. Here we will only be concerned with using the value of ϵ for the determination of $\pi:A$ curves.

The modulus ϵ which is frequency dependent can be measured accurately for many soluble monolayers. For insoluble monolayers it can be determined directly from the slope of the $\pi:A$ curve. Soluble monolayers at high frequencies behave as insoluble monolayers because

for each concentration of surface active material in the bulk there exists a frequency above which surface to bulk diffusional interchange is negligible (Luccassen *et al.* 1972).

If the modulus dependence is determined for a wide range of frequencies experimentally then it is seen that at high frequencies the modulus becomes independent of the frequency. The corresponding surface pressure remains unaltered showing that there is no adsorption from the bulk of the surface. The elasticity then assumes a limiting value ϵ_0 which is given by

$$\epsilon_0 = + \frac{d\pi}{d\ln\Gamma} \quad 4.13$$

where Γ is the surface concentration.

It has been shown by Luccassen (1975) that the shape of the ϵ_0 vs. π curve is influenced much more by the surface to bulk distribution coefficient than the π vs. $\log C$ curve where C = bulk concentration. Thus the shape of the ϵ_0 vs. π curve is more interaction dependent than the π vs. $\log C$ curve. To obtain the required value of ϵ_0 the dilatational modulus must be measured for a wide range of frequency spectrum. Fig. 4.2 for dodecyl triethylene glycol is an example of such an experiment. Here ϵ is plotted against frequency on a bilogarithmic scale and each line provides one extrapolated value of ϵ_0 . Hence from a series of modulus measurements ϵ_0 vs. π can be plotted.

These values are then used to determine the surface concentration by integration starting at high adsorption. Thus

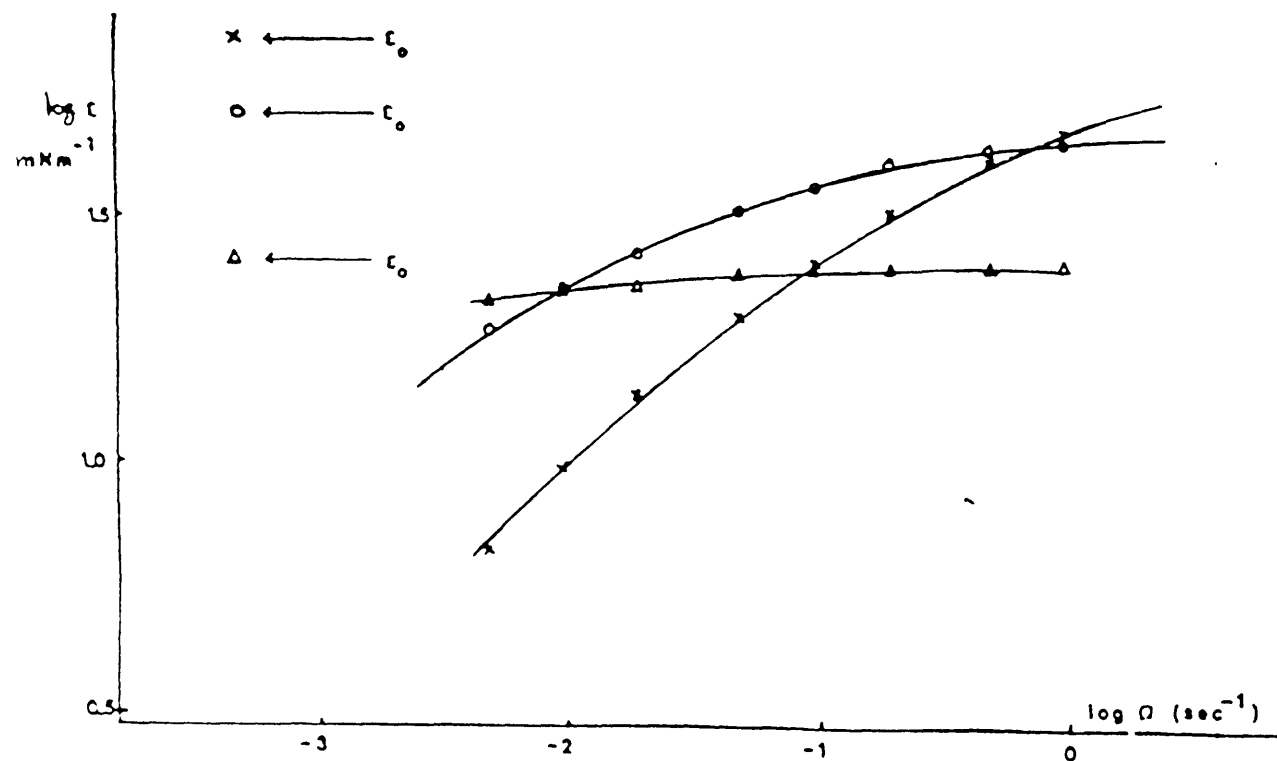


Fig. 4.2 Example of surface dilatational modulus vs. frequency curves. Dodecyl thiethylene glycol (C_{12}E_5) solutions.

$\Delta = 10^{-9}$ mole/ cm^3 ; $O = 5 \times 10^{-9}$ mole/ cm^3 ; $X = 2 \times 10^{-8}$ mole/ cm^3 . Abscissa: $\log \nu$ (ν is frequency in Hertz). Ordinate: $\log_{10} \epsilon$ (mNm^{-1}). Drawn lines: theory below C.M.C. (Lucassen, 1975.)

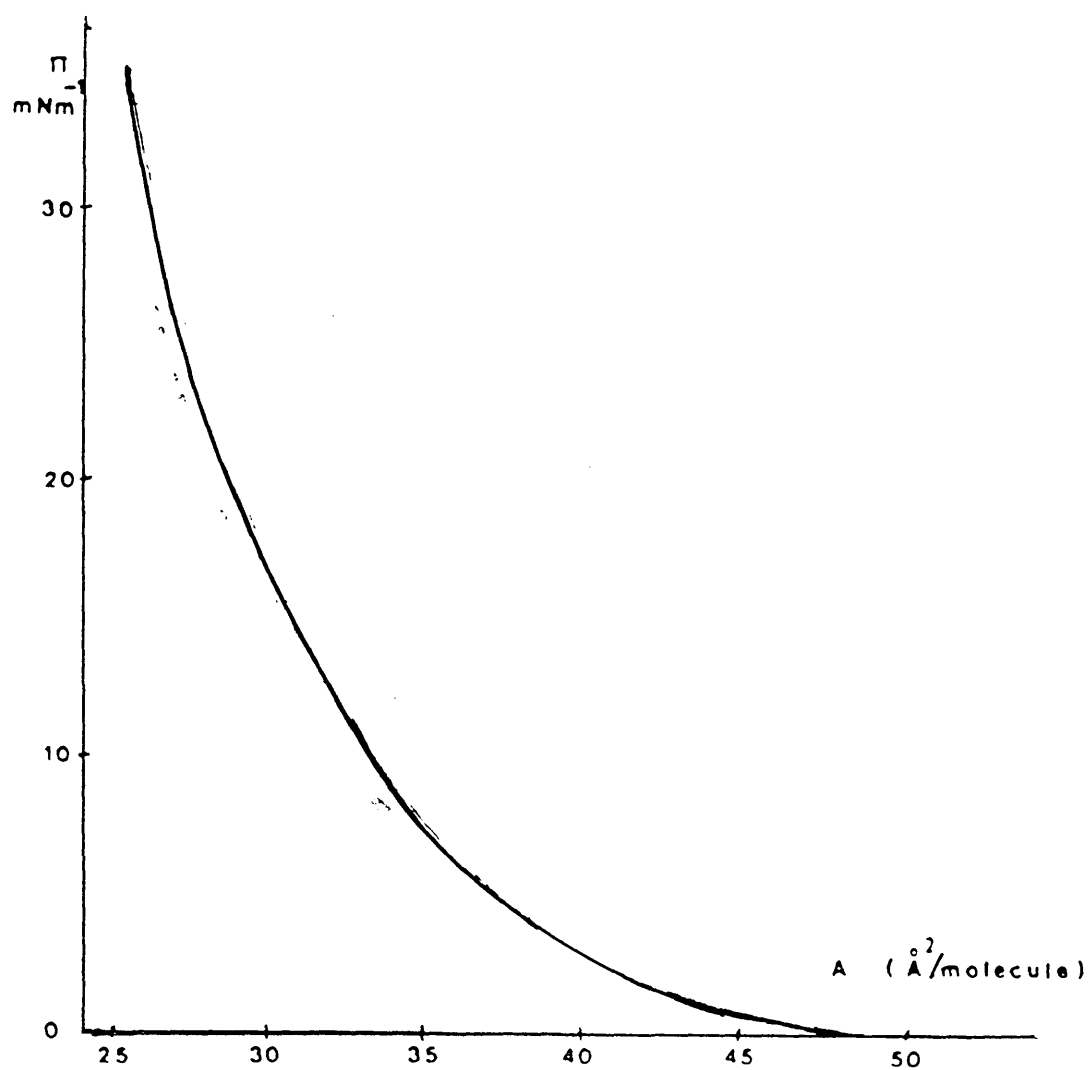


Fig. 4.3 π vs. A curve, obtained by integrating ϵ_0 vs. π curve. Surface coverage obtained from π vs. $\log c$. (Luccassen, 1975.).

$$\ln \Gamma_2 / \Gamma_1 = \int_{\pi_1}^{\pi_2} \frac{1}{\epsilon_0} \quad 4.14$$

As ϵ_0 only gives information on relative adsorption, therefore, one absolute value of surface concentration has to be determined.

From these, $\pi:A$ curves can easily be deduced using the relationship

$$\Gamma = \frac{1}{A} \quad 4.15$$

where A is the area occupied by the molecule.

The π vs. A curve obtained by integrating the ϵ_0 vs. π curve for dodecyl triethylene glycol is given in Fig. 4.3. ϵ_0 values for protein solution were also determined by the extrapolation technique from the ϵ vs. frequency plot as shown in Fig. 4.4. The absolute value of surface concentration for the integration was obtained from the experiments using the radio tracer method. The values of surface concentration thus obtained were compared with those obtained directly by the radio tracer method. It is seen that the values of surface concentration obtained by the two different methods are comparable within the experimental error at both the air/liquid and liquid/liquid interfaces. Fig. 4.5 shows the values obtained at the air/water interface by Gibbs method, integration method and the radio tracer method. The values obtained by using Gibbs equation are comparable only at low concentrations.

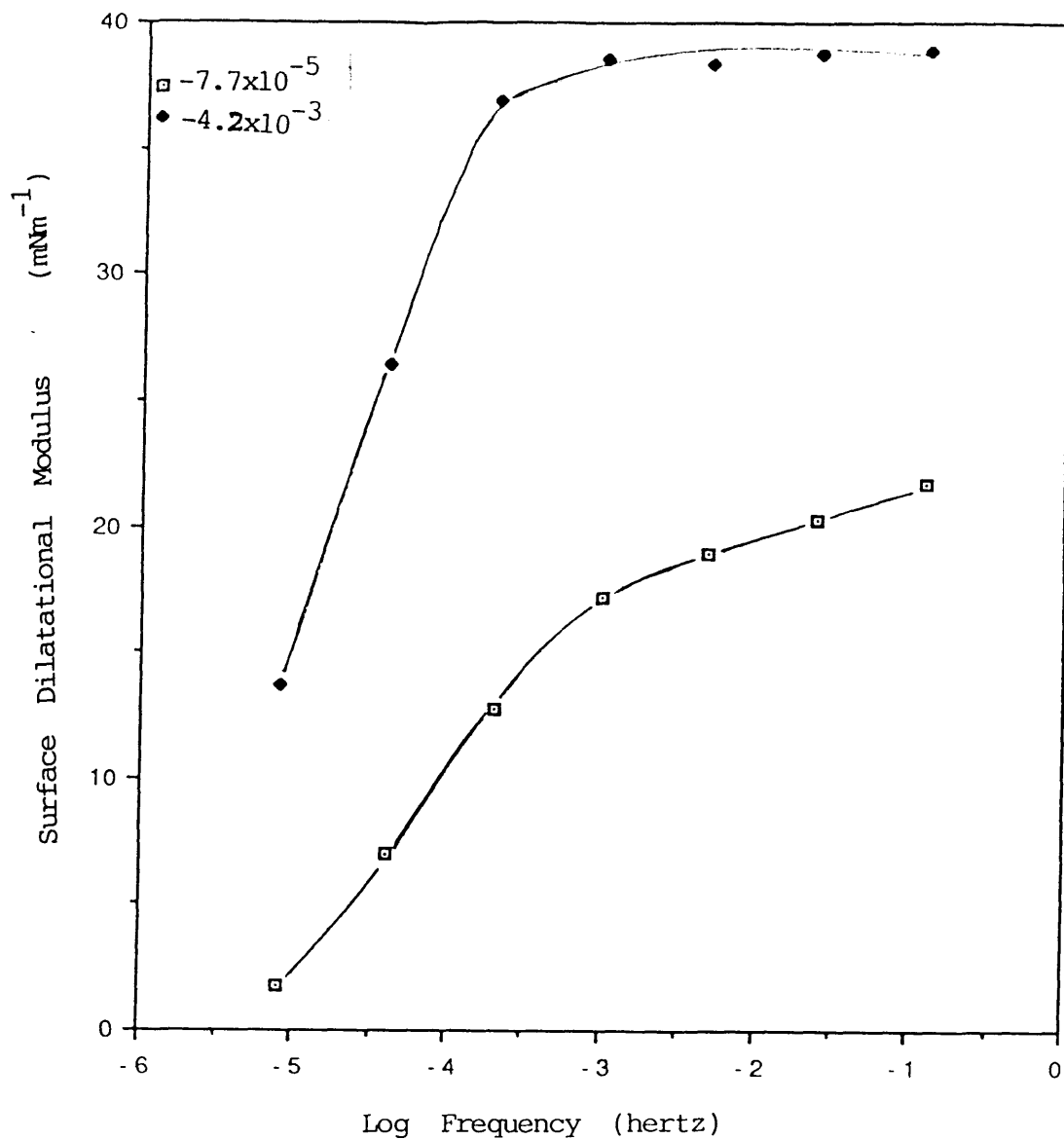


Fig. 4.4 Values of ϵ are plotted against log frequency for protein solution having bulk concentration = 7.7×10^{-5} wt% and 4.2×10^{-3} wt%. ϵ_0 values are obtained by extrapolating the graph..

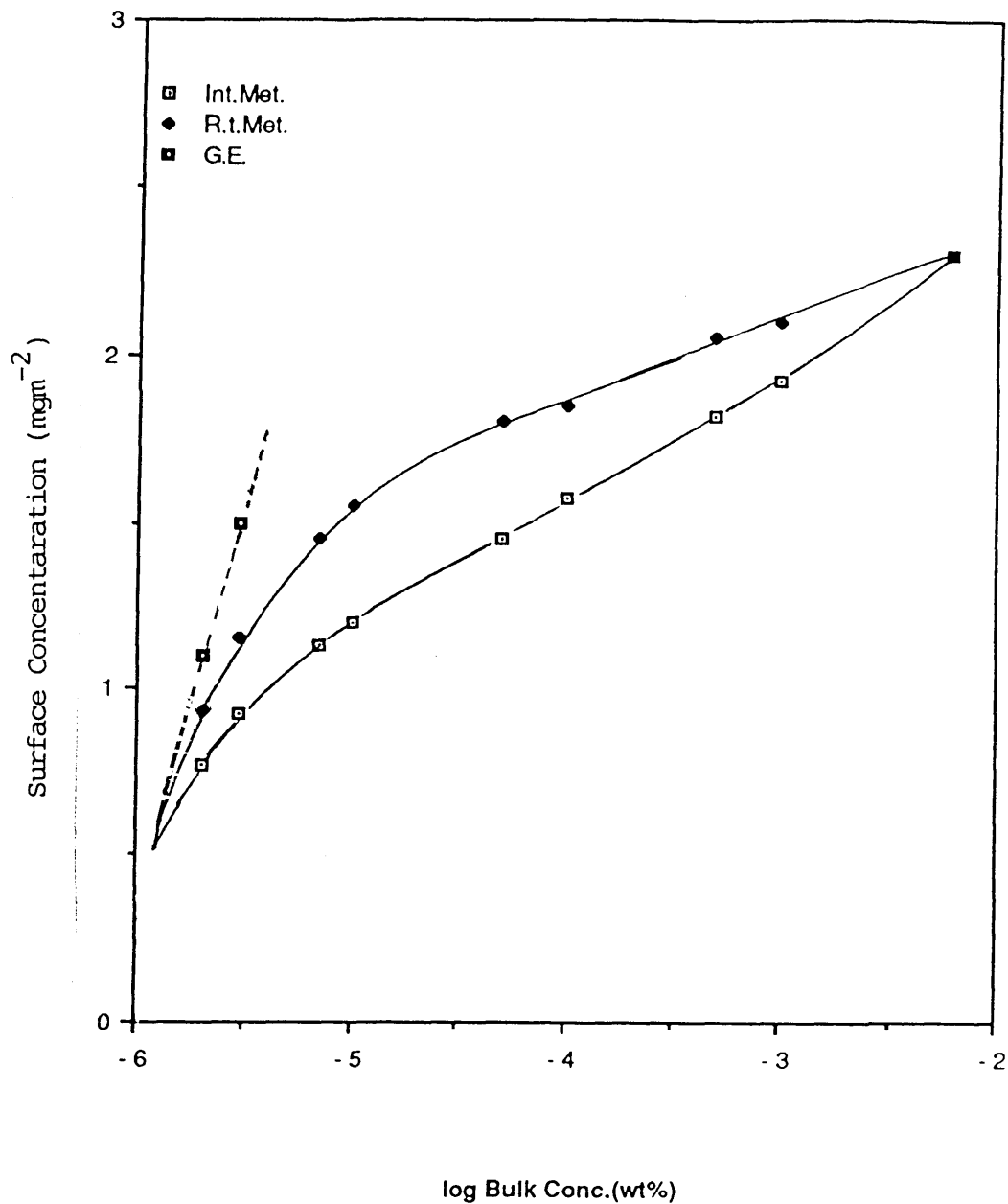


Fig. 4.5 Comparison of the surface concentration measurements obtained by the radio-tracer method and the integration method. Dotted line shows the values obtained by Gibbs' adsorption equation. All the above observations are for protein solution at pH 7.4 at the air/liquid interface.

4.3 EXPERIMENTAL DETERMINATION OF SURFACE CONCENTRATION AT THE AIR/ WATER AND OIL/WATER INTERFACES

4.3.1 Apparatus

The apparatus used consists of a windowless scintillation counter system. The windowless system has been used so that the losses due to the windows of Geiger counters and Alpha scintillation counters are eliminated resulting in a high degree of counting efficiency. The equipment consists of a scintillation counter type 663A and a drawer unit type 723. The standard end cap is removed from the viewing end of the scintillation counter and is replaced with a special flanged end cap which attaches to the drawer unit. The equipment was purchased from Nuclear Enterprises Ltd. The sample to be measured is placed in a petri dish (40mm dia.) which is carried in a light tight drawer. The petri dish is placed in a recess in the drawer in order to ensure that there is no possibility of the sample being brushed against, by any part of the unit whilst the drawer is being pushed in or pulled out. The scintillation counter is attached to the Scaler-Ratemeter SR3 also purchased from Nuclear Enterprises Ltd.

4.3.2 Materials

All the ^{14}C labelled radionuclides used during the experiments and the liquid scintillant used at liquid/liquid interface were purchased from Amersham International plc. and used without further purification.

^{14}C labelled Sucrose, ^{14}C Methylated Bovine Serum Albumin and ^{14}C -Stearic Acid had specific activities of $50\ \mu\text{Ci}/\text{mmole}$, $42\ \mu\text{Ci}/\text{mg}$ and $50\ \mu\text{Ci}/\text{m mole}$ respectively.

For liquid scintillation xylene based Organic Counting Scintillant (OCSTM) was used.

The water, buffers, decane and human serum albumin used throughout the experiments are described in Chapter 3.

4.3.3 Method

Mixtures of different concentrations of HSA and [¹⁴C] methylated BSA solutions, the adsorption of which was to be determined was placed in a petri dish of area ($1.25 \times 10^{-3} \text{ m}^2$) in the drawer of the light tight box. The petri dish was placed directly beneath the photomultiplier tube as shown in Fig. 4.6 so that a constant geometry was obtained for each experiment. At the air/water interface where the aqueous phase was a phosphate buffer, the adsorption of proteins was obtained for a period of 16 hrs at a number of different values of pH. A plastic scintillant was used at this interface. The scintillant was placed at the end of the photomultiplier tube before insertion into its housing unit. The photomultiplier tube with the scintillant in, was then fixed to the drawer unit. To get maximum efficiency the voltage supply to the photomultiplier was adjusted to determine optimum conditions between the background and the sample counting levels.

An aliquot of the labelled protein was then added to the aqueous substrate and allowed to adsorb for a period of 16 hours. As adsorption took place, scintillations were detected by the photomultiplier and the output pulses were coupled to a scaler or ratemeter. They can be counted directly or, if required, could be traced on a recorder or automatically printed out. The pulse rates thus obtained are proportional to the amount adsorbed and were used to determine the interfacial concentration.

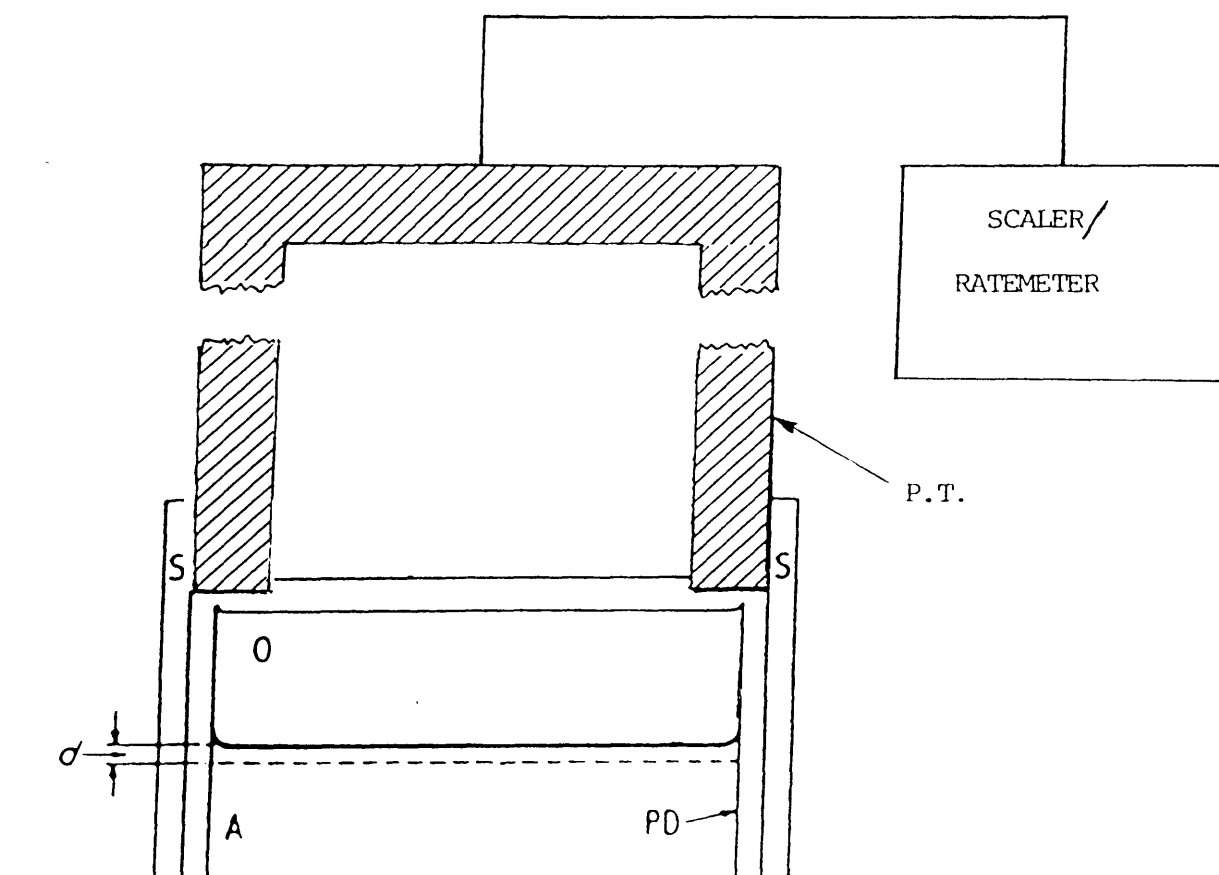


Fig. 4.6 Schematic presentation of the photomultiplier tube placed directly above the petri dish held in a light tight drawer unit. O = oil phase; A = aqueous phase; P.D. = petri dish; $d = 300 \mu\text{M}$ depth; S = steel drawer unit; P.T. = photomultiplier tube.

The method was modified for measurements made at the liquid/liquid interface. Here again the aqueous phase was a phosphate buffer and n-decane was used as the oil phase. An organic liquid scintillant OCSTM which is soluble in n-decane was used instead of the plastic scintillant used at the air/liquid interface. Several concentrations of OCS were tried in order to determine the optimum concentration necessary for a sufficiently high count rate. The voltage supply was also adjusted to give maximum counting efficiency. Adsorption of proteins at liquid/liquid interfaces was thus observed as a function of both bulk concentration and pH. For accurate measurements of surface concentration a number of calibrations and corrections are required. These are outlined in the next section.

4.3.4 Calibrations

The pulse rates obtained during an experiment arise from background and the amounts of radioactive material adsorbed. The background count has to be determined and subtracted. This was done by counting in the absence of radionuclides.

As protein molecules are soluble in the aqueous phase they could not be used to determine the counting efficiency of the equipment. Hence ¹⁴C labelled stearic acid which forms insoluble monolayers was used. The pulse rate obtained as a function of concentration is shown in Fig. 4.7.

Since the range of β particles emitted from ¹⁴C atoms is about 300 μ m in water (Aniansson 1951), the pulse rates obtained from the adsorbed protein films are the sum of the pulse rates from the adsorbed molecules at the interface and from the subphase molecules

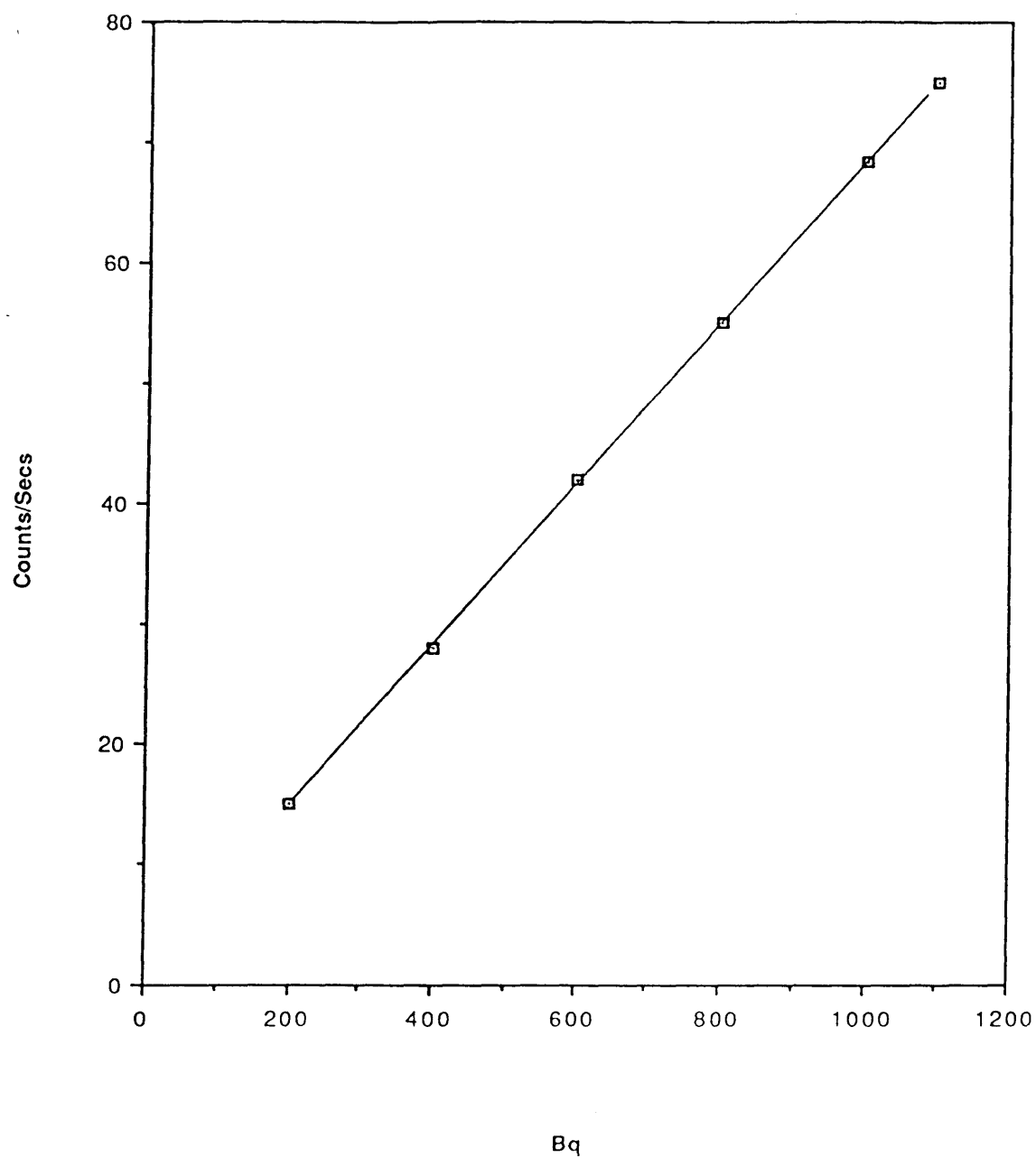


Fig. 4.7 Pulse rate of stearic acid obtained for different concentrations to obtain the counting efficiency of the apparatus, using a plastic and liquid scintilant at the air/liquid and liquid/liquid interfaces respectively.

within a depth of $300\mu\text{m}$ of the interface. In order to distinguish the count rates due to the subphase molecules, a non-surface active material labelled with a ^{14}C atom such as ^{14}C -sucrose which is soluble in the aqueous phase was used. The count rate for sucrose as a function of concentration is shown in Fig. 4.8.

In this way the concentration of the adsorbed protein layer can be determined. The counts per second (CPS) are obtained from

$$\text{CPS} = E_s A_c m_s + E_b A_c m_b \quad 4.16$$

where

E_s is the counting efficiency for material at the interface;

A_c is the specific activity of albumin;

m_s is the concentration of the material at the surface;

E_b is the counting efficiency for material in the bulk;

m_b is the concentration of the soluble material in the bulk.

The experimental reproducibility is $\pm 5\%$. A relatively large source of error is the background count which becomes more significant at higher bulk concentrations. Coincidence counting would improve this.

For measurements at the oil/liquid interface it was seen that the presence of liquid scintillants tends to change the equilibrium at the interface and hence the CPS values decrease with time. This effect was only observed with protein solutions and is not seen with the solutions used for calibration. To ascertain the correct values of Γ the decay rate of CPS were obtained and extrapolated to the time $t = 0$ when the liquid scintillant is added.

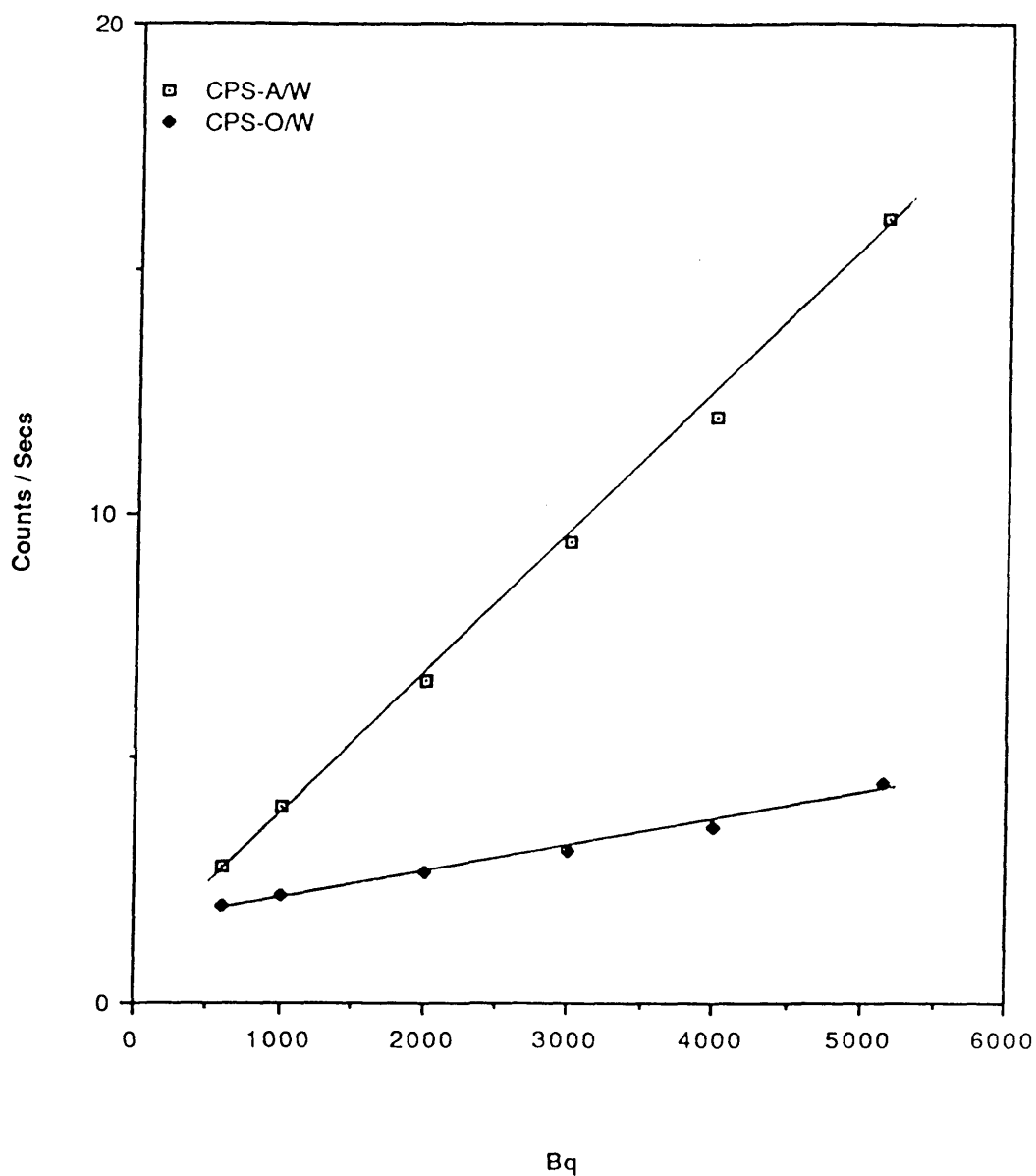


Fig. 4.8 Pulse rate of sucrose obtained for different concentrations at both the air/liquid and liquid/liquid interfaces. Being non surface active, measurements determine the counts for a depth of $300\text{ }\mu\text{m}$, this enables to distinguish the count rates due to the subphase molecules.

4.4 RESULTS

For any particular value of the bulk concentration the values of surface concentration (Γ) measured by this technique during the adsorption of the proteins indicate that the adsorption process is fast as compared to the rate at which the surface pressure (π) reaches its steady state value. This may be seen from Fig. 4.9.

The adsorption isotherm and the surface pressure isotherm at different values of bulk concentration at both the air/liquid and oil/liquid interface are given for pH4.7, pH7.4 and pH11 in Figs. 4.10, 4.11, 4.12 and 4.13 respectively.

From the isotherms it is clear that although the surface or interfacial pressures reach a saturation value the interfacial concentration increases monotonically at both the air/liquid and liquid/liquid interfaces for all the observed values. This is thought to be due to changes in packing of the adsorbed molecules.

It is seen that the surface pressure at the liquid/liquid interface is greater than the corresponding value at the air/water interface for the same bulk concentration although surface concentration is greater at the air/water interface. Thus HSA is more effective at reducing the tension of an oil/liquid interface than of an air/liquid interface. This observation is consistent with observations made by Graham & Phillips (1979).

From the isotherms obtained at different values of pH, it is clear that HSA at both the air/liquid and liquid/liquid interface is most effective at reducing the surface tension at pH4.7. This shows that maximum adsorption takes place at the isoelectric point and can be seen by comparing π values at a given bulk concentration for

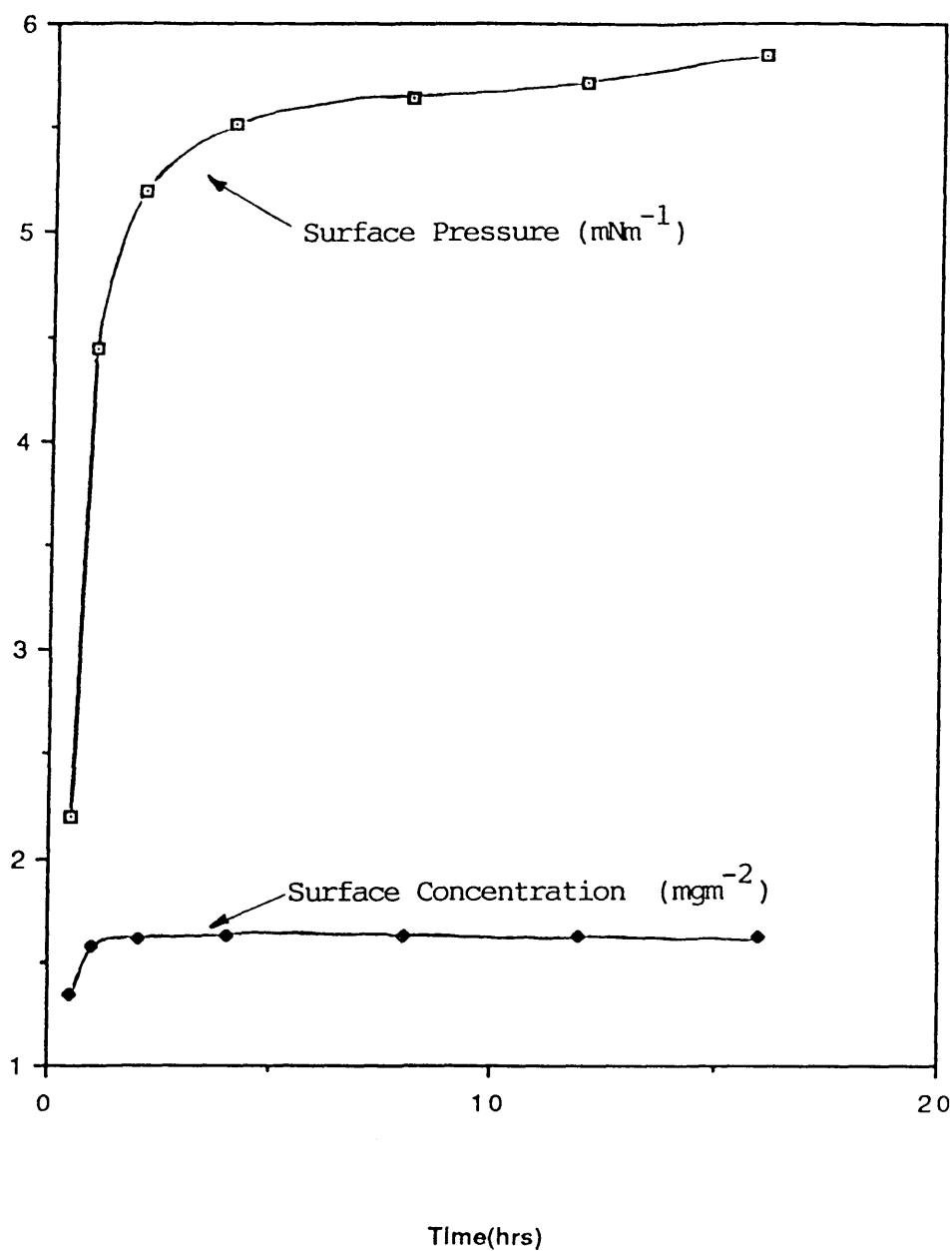


Fig. 4.9 Shows the rate of change of surface pressure and the surface concentration for a bulk concentration = 1.5×10^{-5} wt% at pH7. It is seen that surface concentration reaches a steady state much more quickly than the values for the surface pressure which gradually attain a steady state condition.

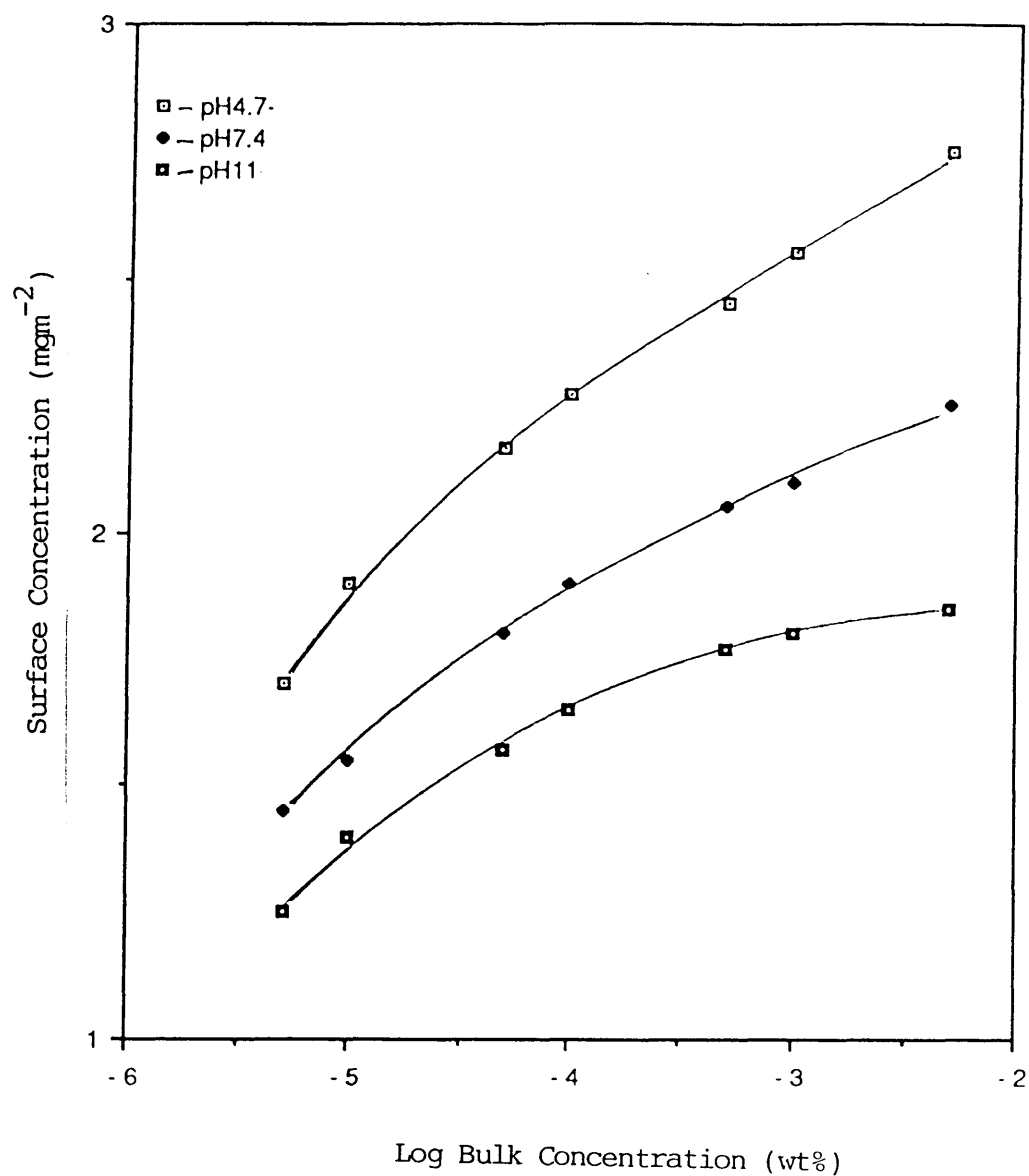


Fig. 4.10 Adsorption isotherm for HSA at PH4.7, 7,4 and 11 are given for different bulk concentrations at the air/liquid interface. The adsorption is maximum at pH4.7, the isoelectric point where the molecule is in its most compact form and least at pH11 where it exists in an extended state.

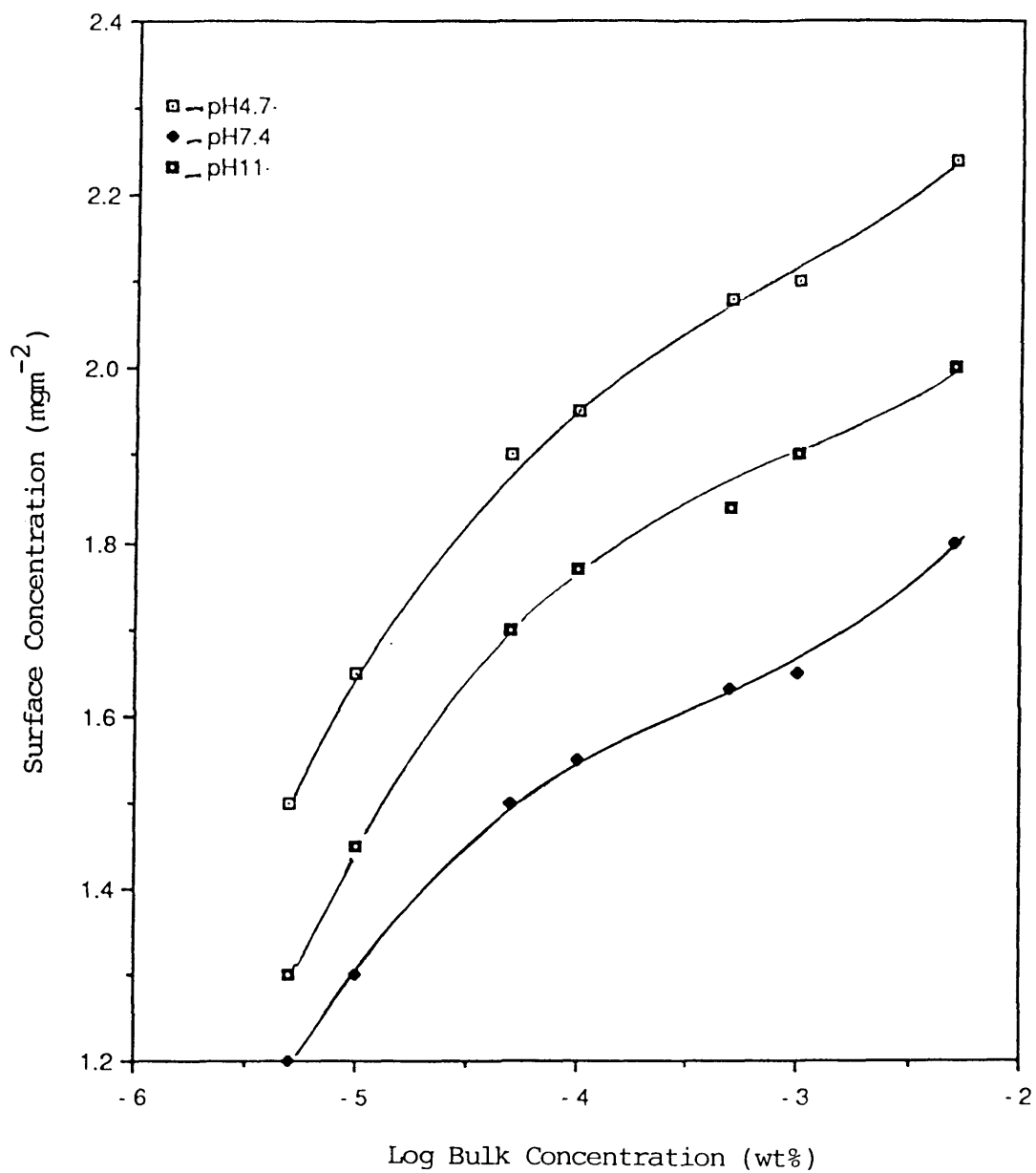


Fig. 4.11 Adsorption isotherm for HSA at pH4.7, 7.4 and 11 are given for different bulk concentrations at the oil/liquid interface. The adsorption here also is maximum at the isoelectric pH but is least for pH7.4 where the molecule is most hydrophilic.

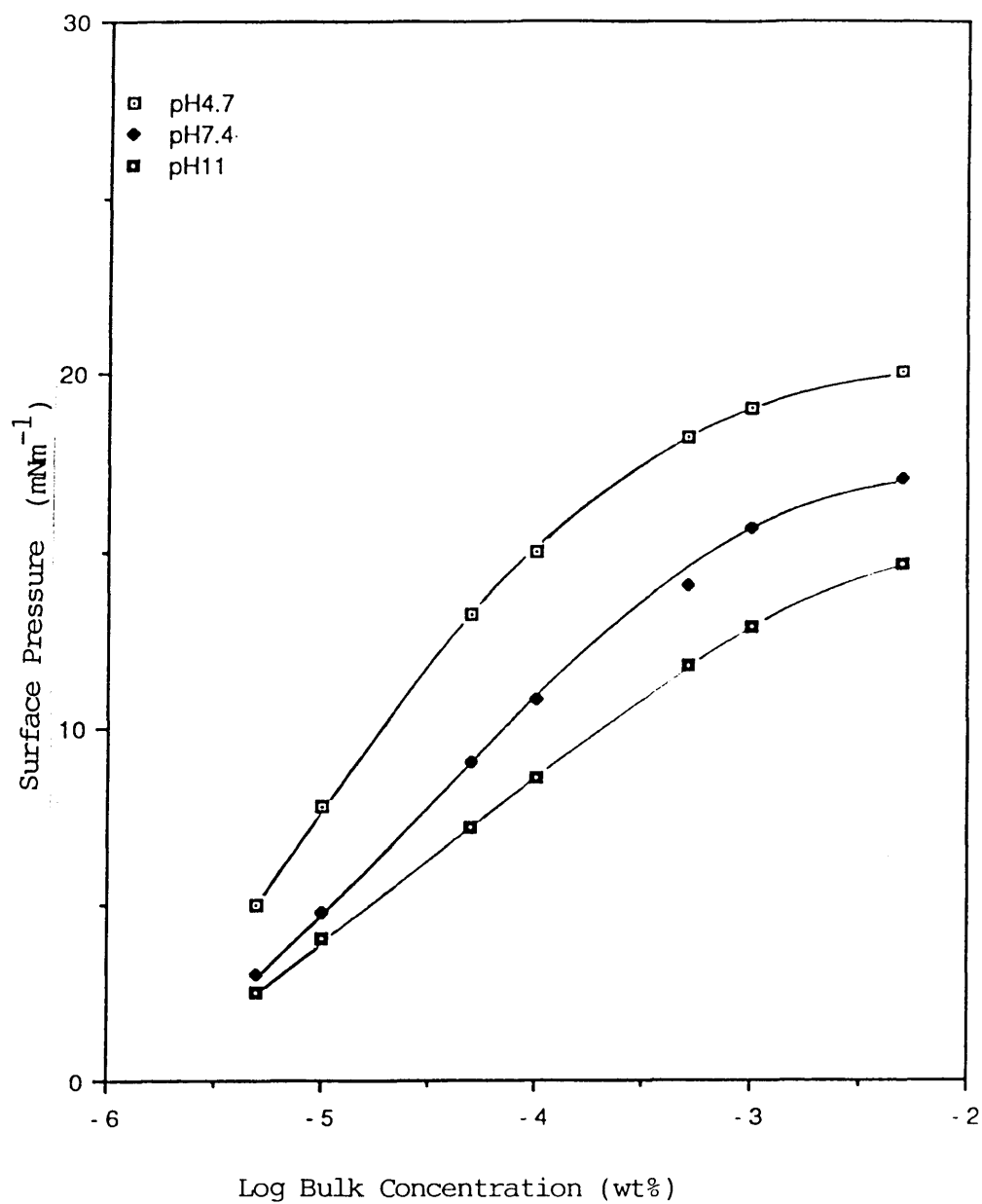


Fig. 4.12 Changes in surface pressure with bulk concentration are shown for pH 4.7, 7.4 and 11 at the air/liquid interface. The isotherms for all the values of pH are seen to reach a steady state at higher bulk concentrations.

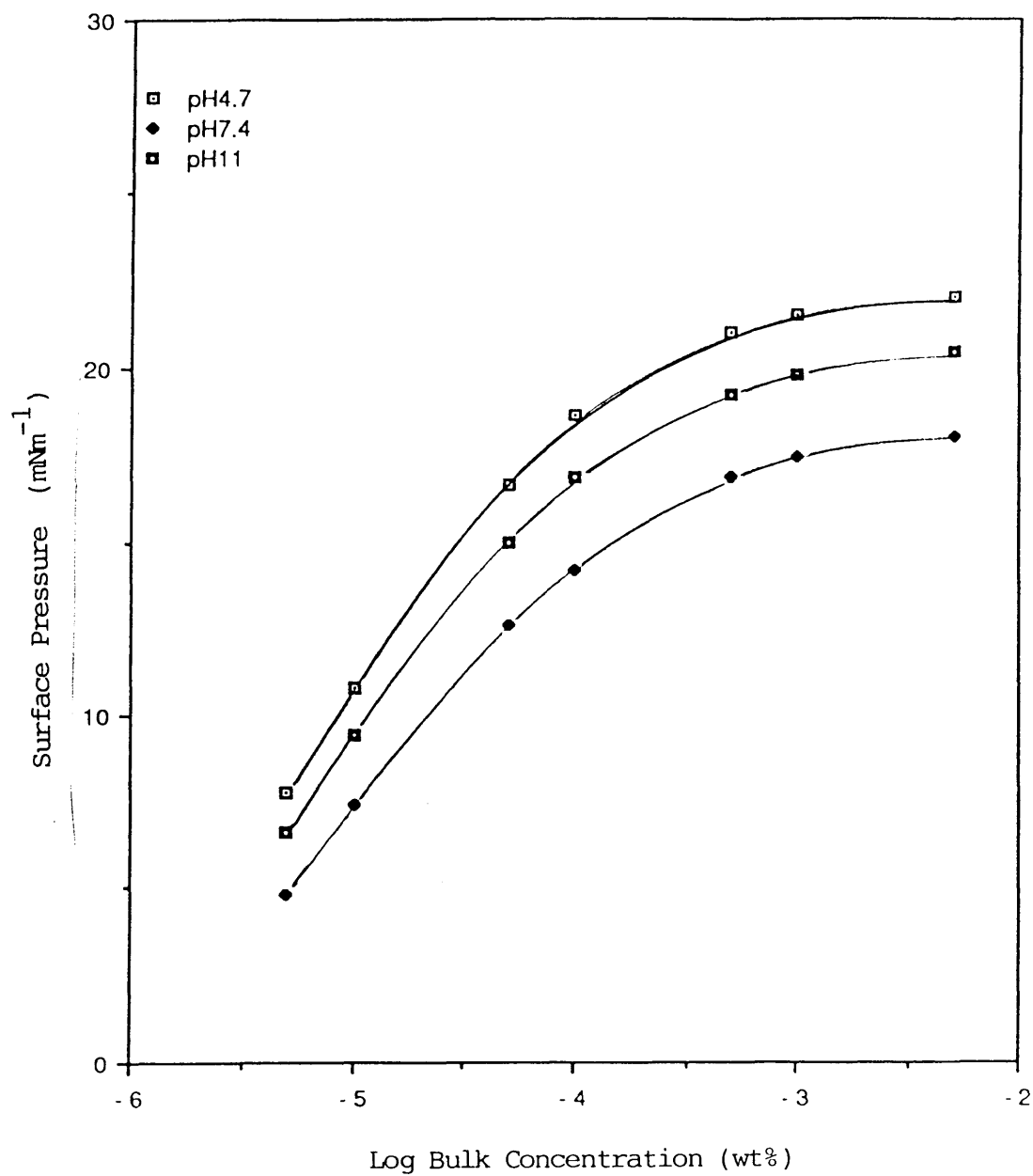


Fig. 4.13 Changes in surface pressure with bulk concentrations are shown for pH4.7, 7.4 and 11 at the oil/liquid interface. The values are higher than the values for the isotherm at the air/water interface. Here also they reach a steady state at higher bulk concentrations.

different pH values. At the air/liquid interface, as the bulk concentration increases, it is seen that the corresponding values of adsorption and surface pressure isotherm are in the order of $\text{pH}4.7 > \text{pH}7.4 > \text{pH}11$. However at the liquid/liquid interface the isotherms are in the order $\text{pH}4.7 > \text{pH}11 > \text{pH}7.4$ indicating that the adsorption of proteins at pH11 is greater than that at pH7.4.

Since proteins carry ionizable groups, alteration in the value of pH are likely to cause changes in the tertiary structure of the molecule in solution. Such changes in structure are likely to influence the way in which the molecules adsorb and hence the surface concentration. Differences in the order of the isotherms at air/water and oil/water interfaces indicate also that the adjacent medium has a role to play. These observations indicate that a detailed study of interfacial phenomena involving adsorbed layers of protein as a function of pH would help in an understanding of the conformational changes which are possible.

Combining the surface pressure data with surface concentration data it is possible to construct $\pi:A$ curves using Eq. 4.15. These curves for air/liquid and liquid/liquid interfaces are shown in Figs. 4.14 and 4.15 for different values of pH.

It is clear from the $\pi:A$ curves at the air/liquid interface that it is most condensed at pH4.7 and the degree of condensation is in the order of $\text{pH}4.7 > \text{pH}7.4 > \text{pH}11$. However, at the liquid/liquid interface the degree of condensation for the different values of pH is in the order $\text{pH}4.7 > \text{pH}11 > \text{pH}7.4$.

On comparison of the $\pi:A$ curves obtained at air/liquid and liquid/liquid interface it is seen that the $\pi:A$ curves at the air/

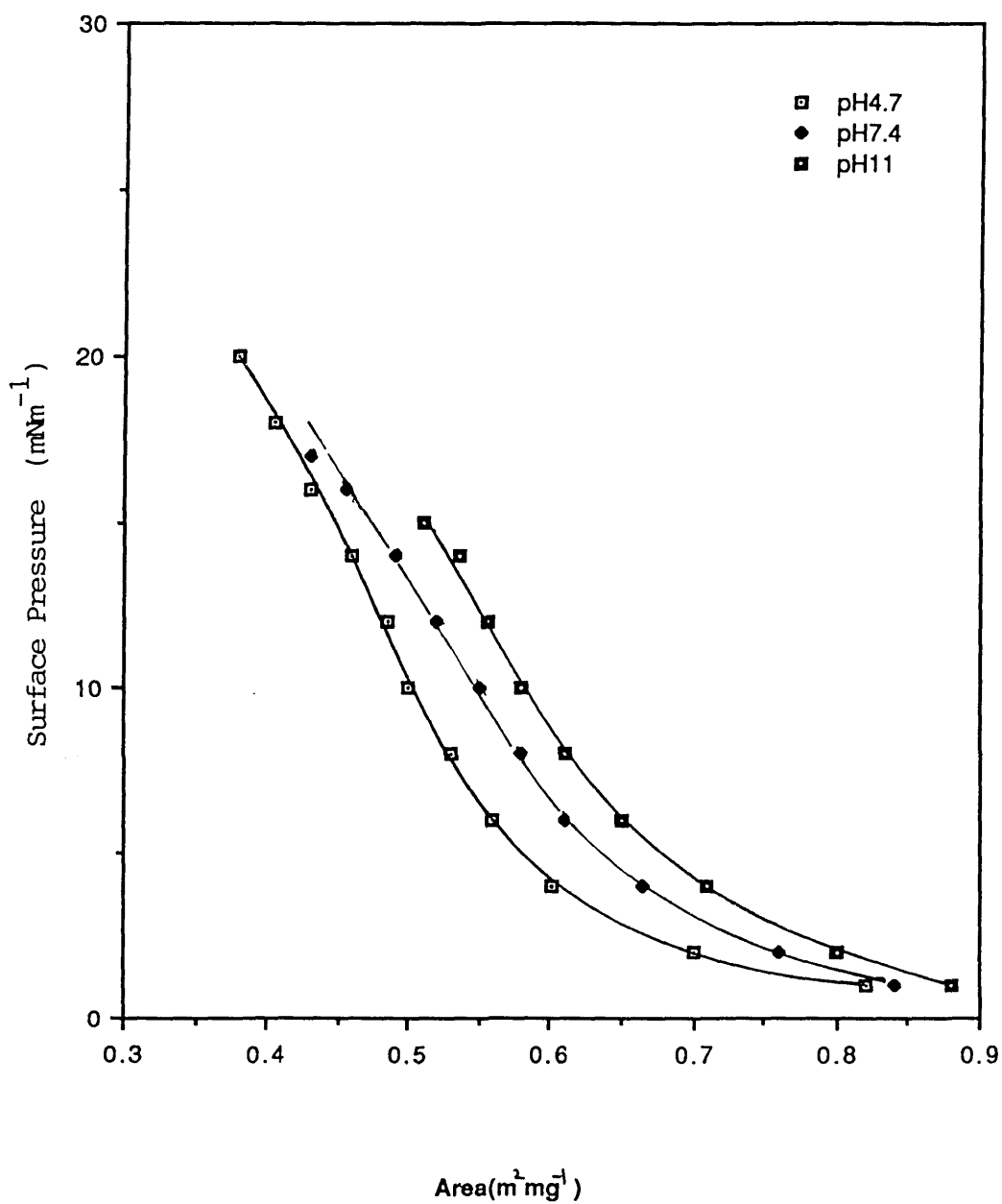


Fig. 4.14 Changes in surface pressure with area are shown for pH4.7, 7.4 and 11 at the air/liquid interface. The curves are most condensed at pH4.7 and expanded at pH11.

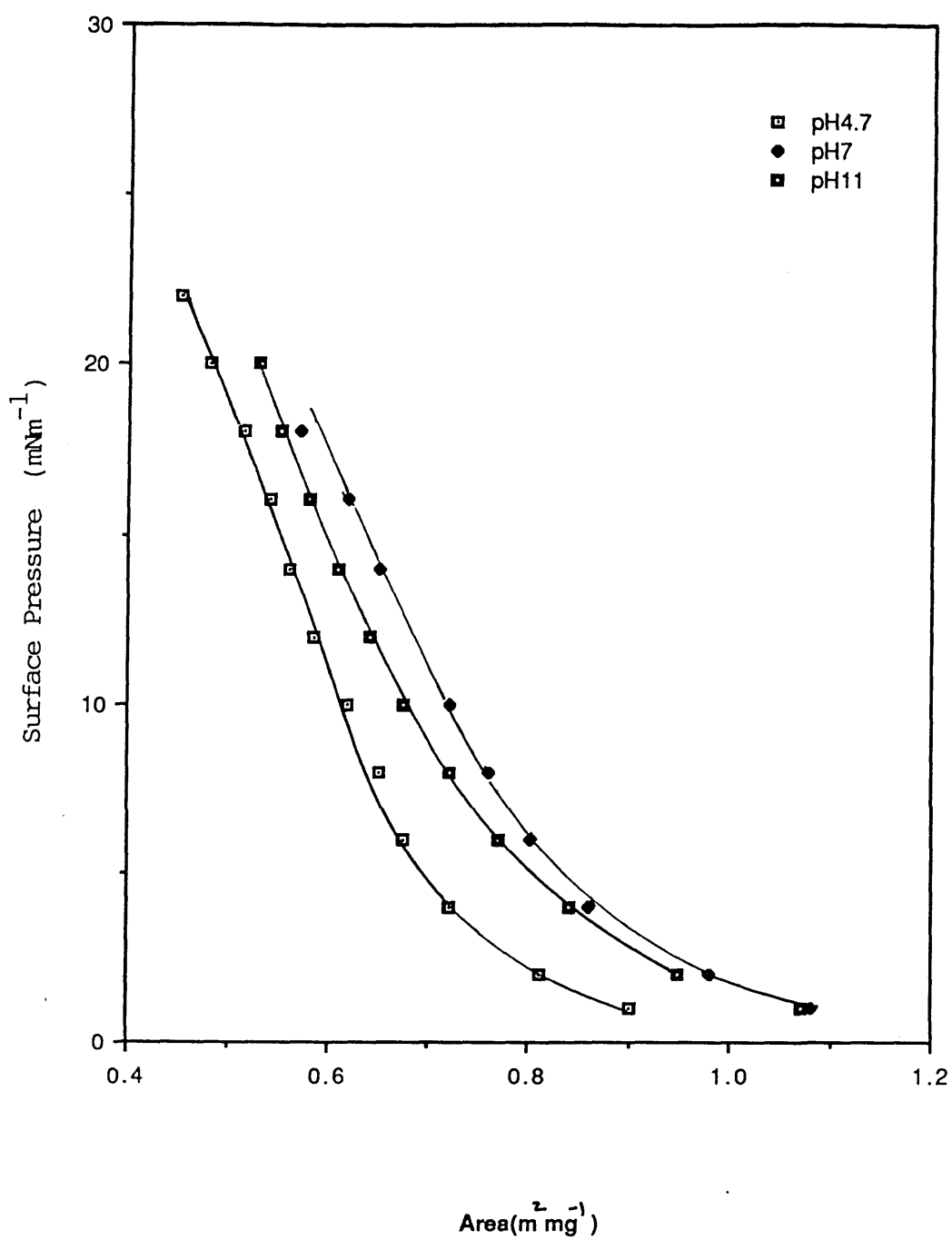


Fig. 4.15 Changes in surface pressure with area at oil/liquid interface are given for pH4.7, 7.4 and 11. Here the curves are most condensed at pH4.7 and expanded at pH7.4.

liquid interface are more condensed than those obtained at the liquid/liquid interface for all the three different values of pH.

These isotherms are then analysed to determine the conformation of the adsorbed protein molecule with changes in pH. This is achieved by comparing the curves obtained with those of linear flexible polymers which have been calculated from a surface equation of state based upon statistical theories of polymers. In this way, the conformation of the protein films at both the air/liquid and oil/liquid interfaces can be evaluated in terms of protein segments lying in the surface or being solvated by either of the bulk phases.

4.5 MODELS OF ADSORBED POLYMER FILMS

Several theories relating to the surface pressure of adsorbed molecules with the surface concentration have been developed. Singer (1948) has derived an equation of state for linear polymers adsorbed flat at a liquid surface. He proposed a two dimensional quasi lattice in which the linear polymers could move adopting a large number of configurations. He used a thermodynamical statistical treatment for the surface polymers which is analogous to that of Flory & Huggins (1941) for polymers in the bulk. The equation derived by Singer is:

$$\pi = \pi_o \left[\frac{x-1}{x} \frac{Z'}{2} \ln \left(\frac{1-2\theta_s}{Z'} \right) - \ln(1-\theta_s) \right] \quad 4.17$$

where $\pi_o = \frac{kT}{a_o}$;
 k = Boltzman constant;
 T = temperature;

- a_o = the limiting condensed area per statistical unit of the polymer;
- θ_s = the degree of surface coverage and is given by the ratio $\frac{a_o}{a_1}$ where a_1 is the observed area per statistical unit of the polymer;
- x = number of statistical units per polymer molecule. In the case of proteins x is large and so $\frac{x-1}{x} \rightarrow 1$;
- Z' = the lattice co-ordination number and is designated a value between 4 (when the polymer is completely flexible and can sample any lattice site on the surface) and 2 (when the polymer is a rigid and has a limited choice of lattice site).

For the protein the equation can be written as:

$$\pi = \pi_o \left[\frac{Z}{2} \ln(1 - \frac{2\theta_s}{Z'}) - \ln(1 - \theta_s) \right] \quad 4.18$$

Theoretical π :A curves have been calculated using Eq. 4.18 and are shown in Figs. 4.16 and 4.17 when Z' has values between 2.1 and 4, for arbitrary values of a_o .

The smaller the value of a_o or Z' the more condensed will be the π :A curve. This model by Singer has been further extended by Motomura & Maturua (1963) to account for segment-segment and segment-solvent interactions and by Frisch & Simha (1956, 1957) to account for loop formation when some of the segments are not in direct contact with the surface, the remaining segments forming loops and trains in the liquid phase. The equation derived by Frisch & Simha (1956) is similar to

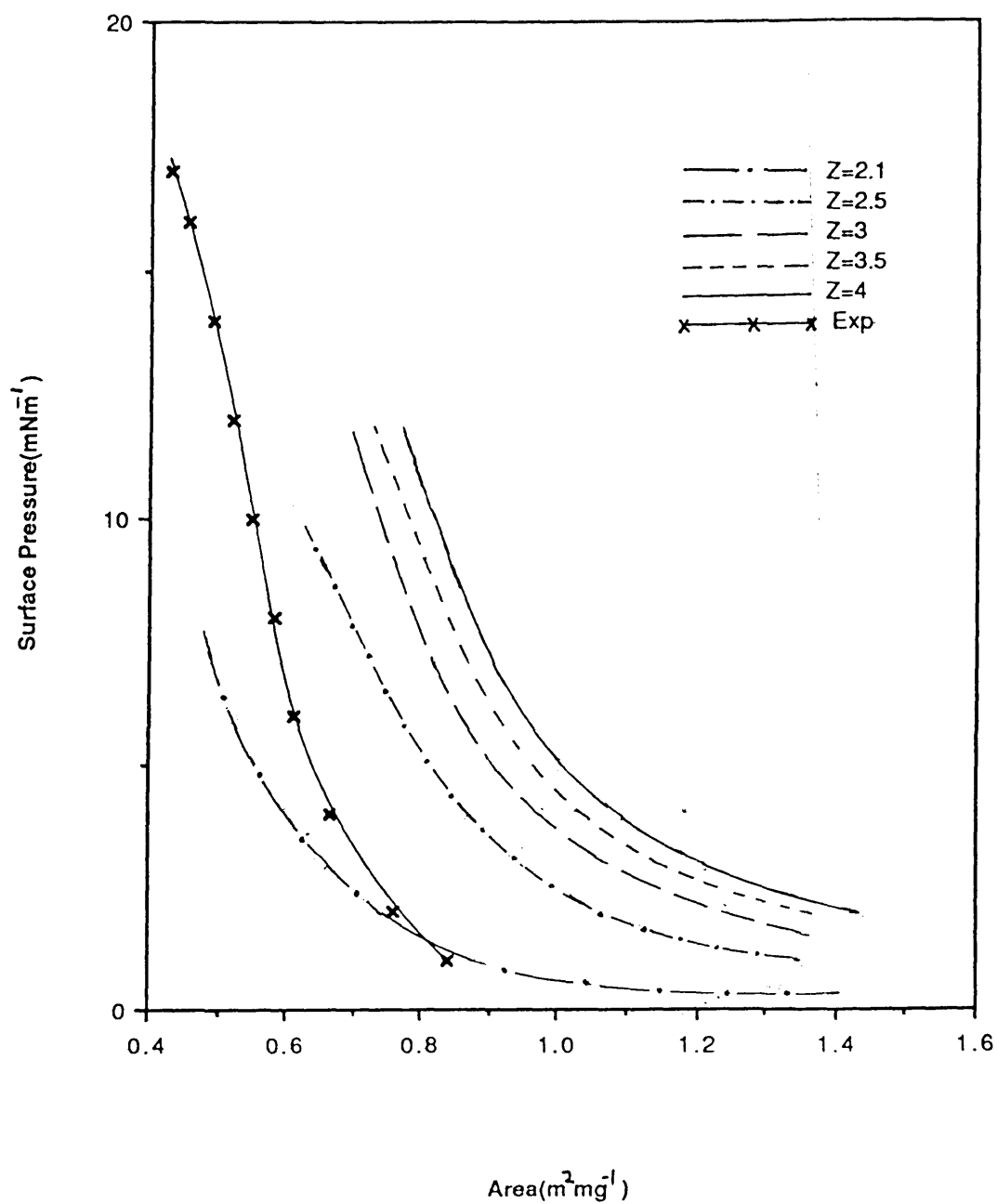


Fig. 4.16 Surface pressure vs. area curves obtained by the application of Singer's equation. Here $a_0 = 10$ and the value of Z' varies between 2.1 and 4. x-x is an experimental curve

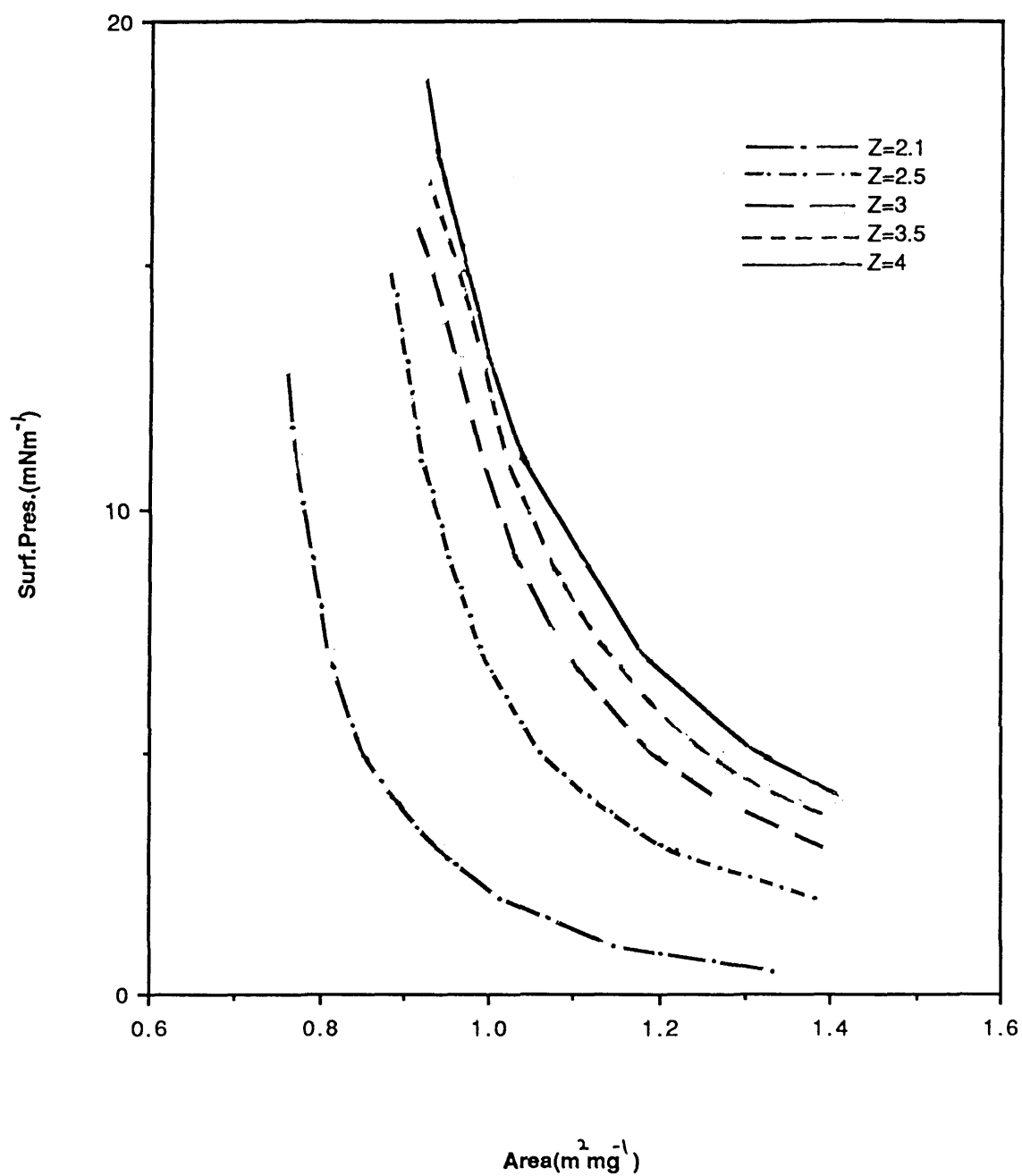


Fig. 4.17 Surface pressure vs. area curves obtained by using Singer's equation. Here $a_0 = 15$ and the value of Z' varies between 2.1 and 4.

Eq. 4.17 as it predicts π when only part of the polymer is in contact with the surface. The equation then becomes

$$\pi = \pi_0 \left[\frac{Z}{2} \frac{\nu_1^{-1}}{\nu_1(1-1/t_1)} \ln \left[1 - \frac{2}{Z} \left(1 - \frac{1}{t} \right) p \theta_s \right] - \ln (1-p \theta_s) \right]$$

4.19

where t_1 = number of amino acid residue/protein molecule;

ν_1 = number of amino acid in direct contact with the surface;

$t - \nu_1$ = in loop configuration;

$$p = \frac{\nu_1}{t_1}.$$

When all amino acid residues are in the train configuration then $\nu_1 = t_1$ and $p = 1$ and this equation becomes identical to that of Singers. π :A curves obtained by using Eq. 4.18 are shown in Figs. 18 and 19.

Silberberg (1962) criticized the theory of Frisch *et al.* (1956) on the grounds that it predicts too high a proportion of long loops. He explained the adsorption phenomenon using a lattice model in which all or only some surface sites are active, i.e. capable of adsorbing. He was the first to introduce a statistical theory for an isolated adsorbed chain consisting of loops and trains. He assumed that the polymers are adsorbed by attaching many segments to the surface and extending short loops away from it. This suggested that the bound fraction tends to be much larger than that proposed by the Frisch & Simha (1956, 1957) model. Silberberg (1968) later modified his theory to take account of lateral interactions between the adsorbed chains. He used a step function to describe interactive loops.

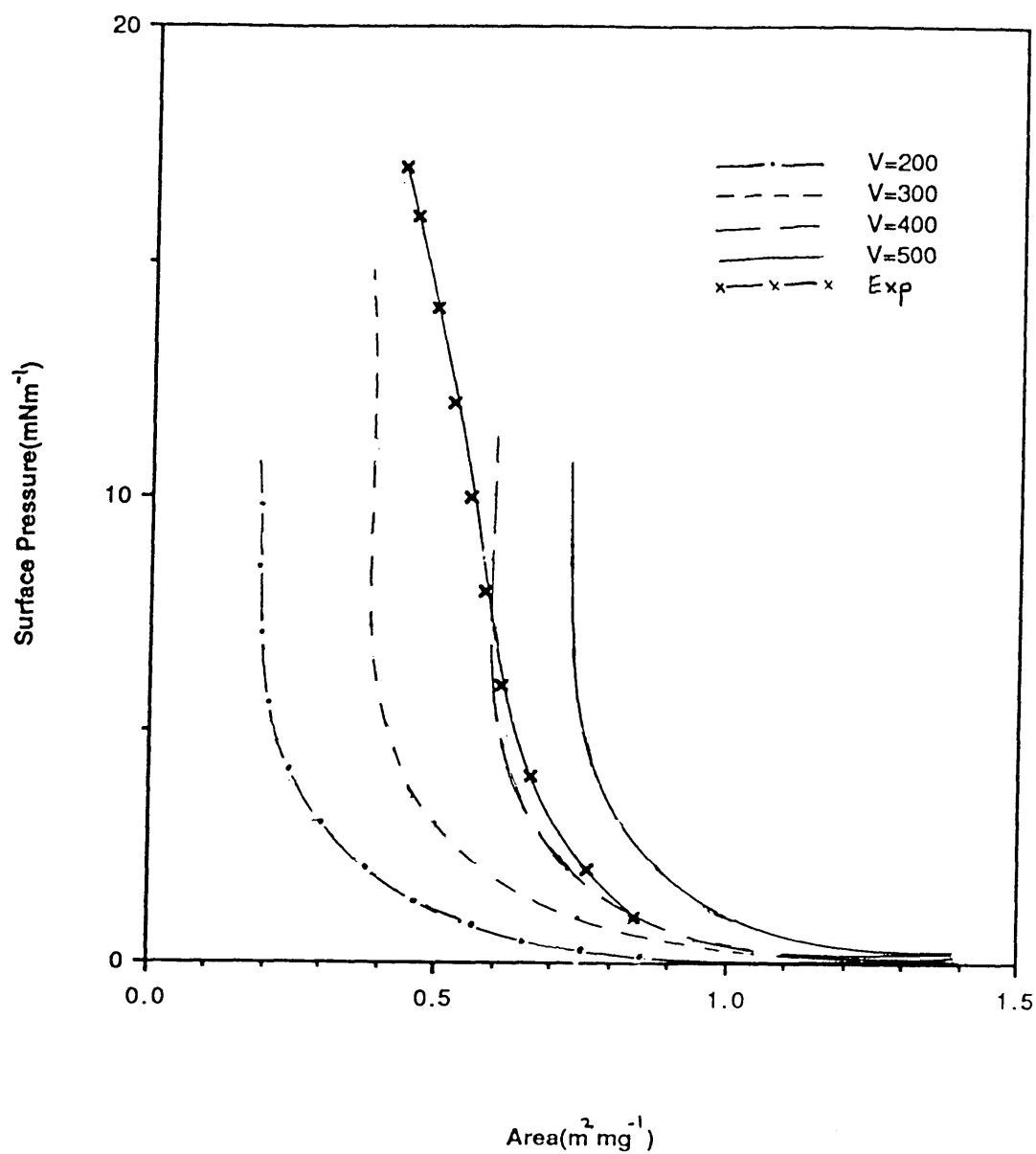


Fig. 4.18 Surface pressure vs. area curves obtained by using the equation derived by Frisch & Simha to account for loop formation. Different values of ν are plotted when $a_o = 15$ and $Z' = 2.1$. x-x is an experimental curve for pH 7.4

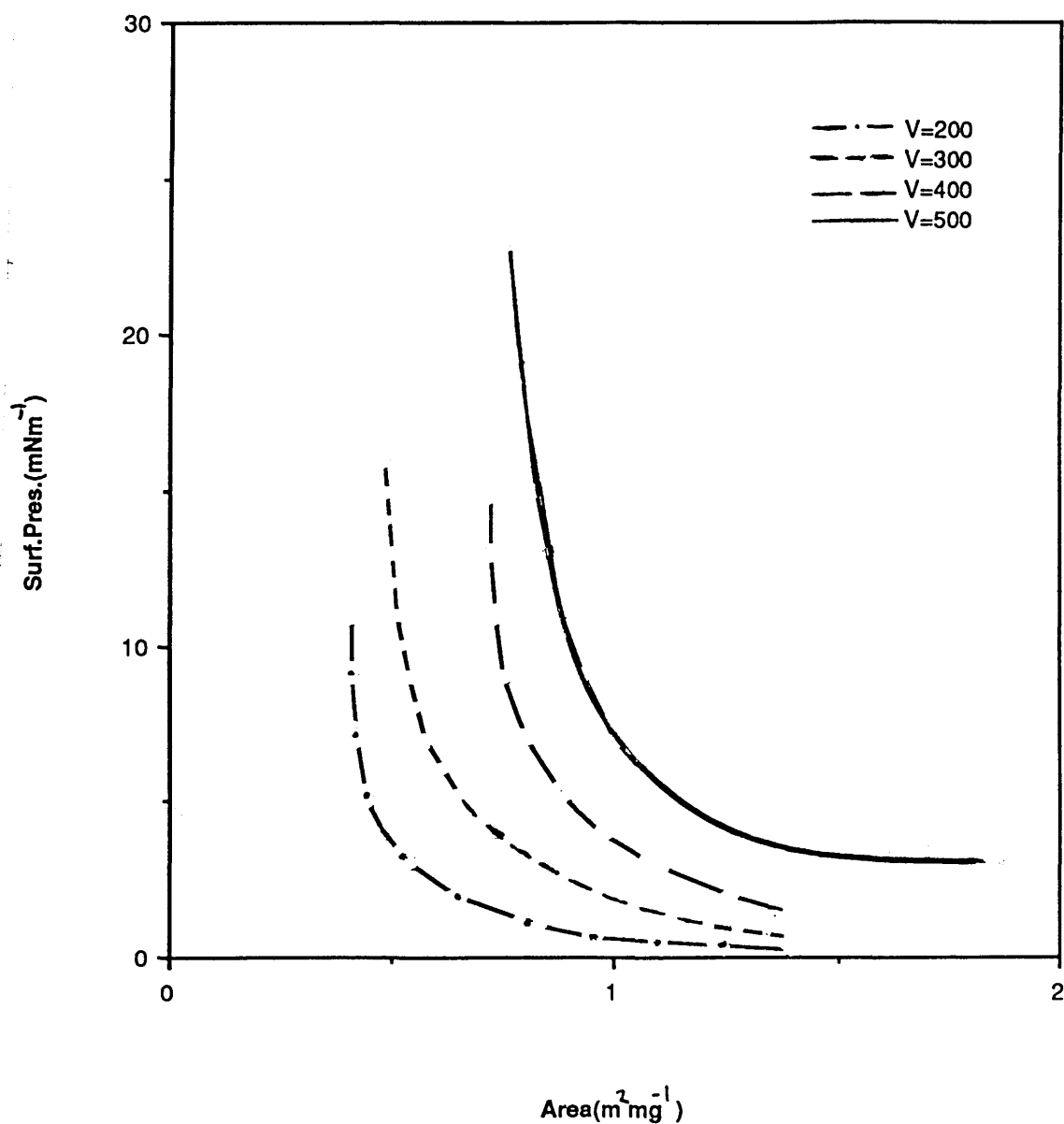


Fig. 4.19 Surface pressure vs. area curves obtained by using the equation derived by Frisch & Simha to account for loop formation. Different values of ν are plotted when $a_0 = 15$ and $Z' = 4$.

Hoeve *et al.* (1965) criticized the original theory of Silberberg on the grounds that the positions of bound segments were too widely spaced and produced a theory in which the loops and train size distribution was narrow and equal. Further, Hoeve (1970, 1971) described the lateral interactions between the polymer chains by introducing an exponential function. Both Silberberg and Hoeve assumed that during adsorption the surface was completely covered by loops and trains but tails were ignored (see Fig. 4.20).

Unlike previous theories, Roe (1974) explained polymer adsorption from solution by using an arbitrarily fixed lattice, parallel to the surface to evaluate the number of ways of arranging polymer and solvent molecules. The lattice was divided into layers of lattice sites labelled $i = 1, 2, 3, \dots, M$. Each layer consists of L lattice sites. Each lattice site is occupied by either a polymer segment or a solvent molecule. Thus

$$N^0 + rN = M_L \quad 4.20$$

where N^0 is the number of solvent molecules and N is the number of polymer molecules with r segments. Roe calculated the number of ways of arranging N^0 solvent molecules and N polymer chains but was not able to determine the loop, tail and train distributions which contributed to the adsorbed polymer chain.

Scheutjens & Fleer (1979, 1980, 1982) were able to derive a more comprehensive theory based on Roe's ideas. They combined Roe's theory for polymer adsorption with the Flory-Huggins theory (1941, 1942) of polymer solution thermodynamics. They took account of

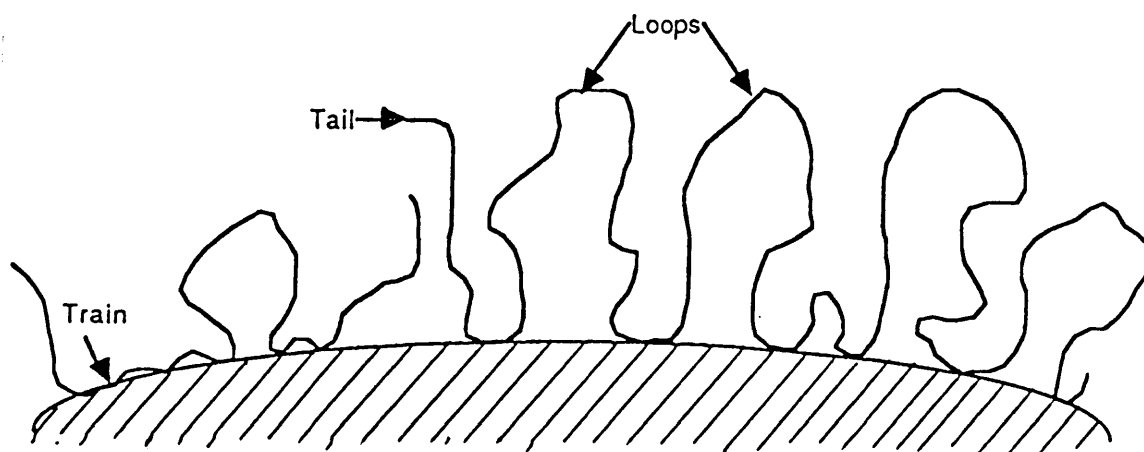


Fig. 4.20 Schematic representation of adsorbed polymer chains on a particle surface.

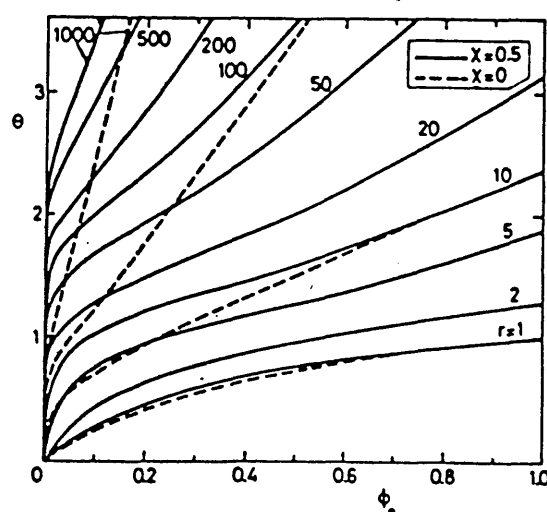


Fig. 4.21 Adsorption isotherms for oligomers and polymers over the whole range of solution volume fractions, from $\phi_* = 0$ to $\phi_* = 1$. The total surface coverage is expressed in equivalent monolayers. The chain length, r , is indicated. Full curves $\chi = 0.5$; dashed curves $\chi = 0$; $\chi_s = 1$; hexagonal lattice ($\lambda_0 = 0.5$) (Scheutjens & Fleer, 1982).

segment-solvent interactions through the Flory-Huggins parameter χ . The interaction between segments and the surface was described in terms of a differential segmental adsorption parameter χ_s .

The variables computed by Scheutjens & Fleer (1982) for polymer adsorption are:

1. The relation between adsorbed amount Γ and the bulk polymer concentration.
2. The fraction of segments adsorbed in trains f ($f = \theta/\Gamma$ where θ_s is the surface coverage).
3. The fraction of segments in loops or tails and their distribution.
4. The adsorbed layer thickness.

To make overall generalizations, it is necessary to predict the variations of the above quantities with surface coverage, molecular weight (m) and the molecular weight distribution (M.W.D.) of the polymer. Scheutjens & Fleer (1982) have determined the above variables as a function of polymer volume fractions ϕ_* , chain length r , and the polymer-solvent interaction parameter χ . Most of their results are for the close packed hexagonal lattice and chain lengths of up to $r = 1000$ were considered. Fig. 4.21 shows the adsorption isotherms of Scheutjens & Fleer (1982) from their theory for the whole concentration range of polymer volume fractions between 0 and 1. The total surface coverage θ_s is expressed in equivalent monolayers. The influence of solvency was also included in these curves by using two values of χ . $\chi = 0$ (athermal solvent) and $\chi = 0.5$ (theta solvent). It can be seen from this graph that oligomers adsorb according to a Langmuir type isotherm. The higher value of r (i.e. the higher the

M.W.) the surface coverage θ_s increases more with ϕ_* . The effect of solvent quality on adsorption is important for dilute polymer solutions. At low ϕ_* the adsorbed amount increases with decreasing solvent quality. The effect becomes more with increasing chain length. This is consistent with the theories of Silberberg (1962, 1968) and Roe (1974) and is due to the natural attraction between segments in poor solvents.

The adsorption isotherm in the dilute concentration region according to Scheutjens & Fler's theory (1982) is shown in Fig. 4.22. Again a range of chain lengths ($r = 1-5000$) are covered. Under these conditions, monomers hardly adsorb. When $r = 10$ the isotherm is just beyond the Henry region (giving some curvature) and for $r > 20$ high affinity isotherms are obtained. The adsorption isotherms for longer chains ($r > 100$) are typical of those observed experimentally for most polymers which are not too polydisperse, i.e. showing a steep initial rise followed by a nearly horizontal pseudo-plateau (which only increases a few per cent per decade of ϕ_*). In dilute solution, the adsorbed amount from θ_s solvents increase with increasing chain length. However, in good solvents θ_s is much smaller and levels off for long chains to reach a plateau which is essentially independent of molecular weight. Variations of bound fraction f (i.e. the fraction of segments in trains) and direct surface coverage θ_1 (i.e. the volume fraction of polymer in the first layer) as a function of chain length for various volume fraction of polymer ϕ_* are shown in Fig. 4.23. In the Henry region ($\phi_* < \phi_*^c$), f is high and is independent of chain length $r > 20$. Scheutjens & Fler calculated that here the molecules lie nearly flat on the surface with 87% of the segments in trains.

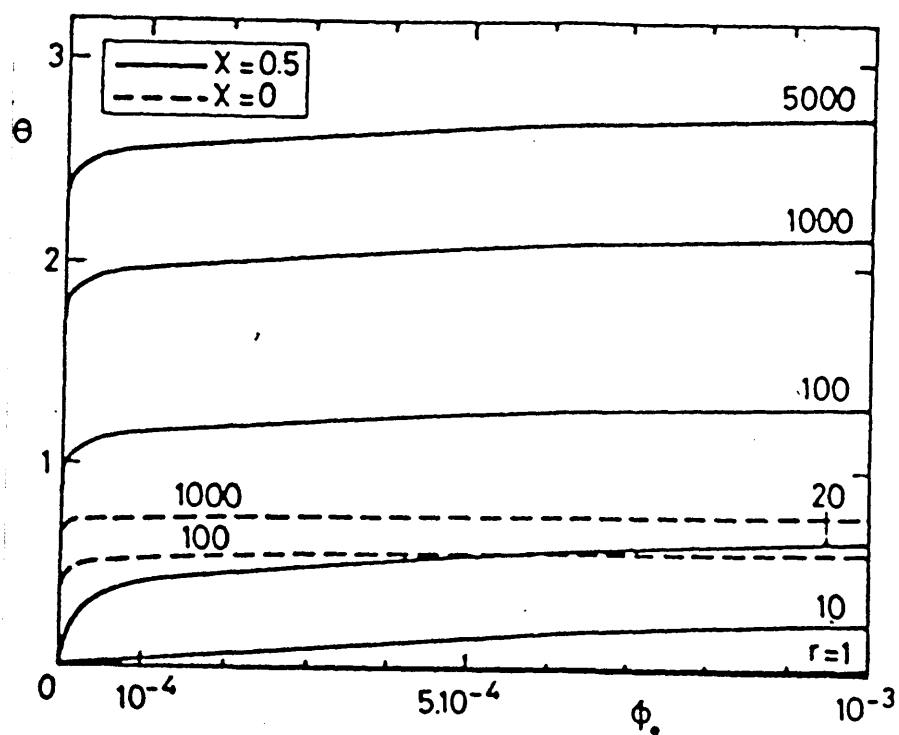


Fig. 4.22 Adsorption isotherms for oligomers and polymers of Fig. 4.21 in dilute concentration region. Full curves $\chi = 0.5$; dashed curves $\chi = 0$; $\chi_s = 1$; hexagonal lattice ($\lambda_0 = 0.5$) (Scheutjens & Fleer, 1982).

At $\phi_{*} = 1$, f is proportional to $r^{-\frac{1}{2}}$. In Fig. 4.23 (b) θ_1 increases sharply with r at short chain lengths. In very concentrated solutions θ_1 is independent of r over the whole range of chain lengths. In good solvents, the direct surface coverage is lower due to stronger repulsion between the segments.

Scheutjens & Fler (1980) used a matrix formalism to calculate the conformation of adsorbed chains. From the information thus obtained they computed the concentration profile due to loops, tails and trains. If the numbers and average lengths of trains, loops and tails are known, the root-mean-square average layer thickness t_{rms} can be calculated.

For testing predictions made by the theory of Scheutjen & Fler it is essential that one should use monodisperse polymers. However, such systems are not very common. Yet qualitative comparison between theory and experimental results have been made. Van der Linden & Van Leemput (1978) reported the results of the adsorption of near monodisperse polystyrene fractions from cyclohexane at 35°C (θ -solvent) and carbon tetra chloride (good solvent) at 35°C on non porous silica. The results showed the expected trend of a sharp rise in the adsorbed amount with increasing polymer concentration reaching a plateau. However, quantitative results are difficult to compare.

De Gennes (1976) proposed a theory for polymer adsorption based on scaling relations for semidilute polymer solutions. Polymer solution can be studied by the local properties (i.e. at strong magnification or the conformations and motions of one monomer inside the chain) or global properties (i.e. at weak magnification or the dependence of physical properties such as on chain length, concentration or few

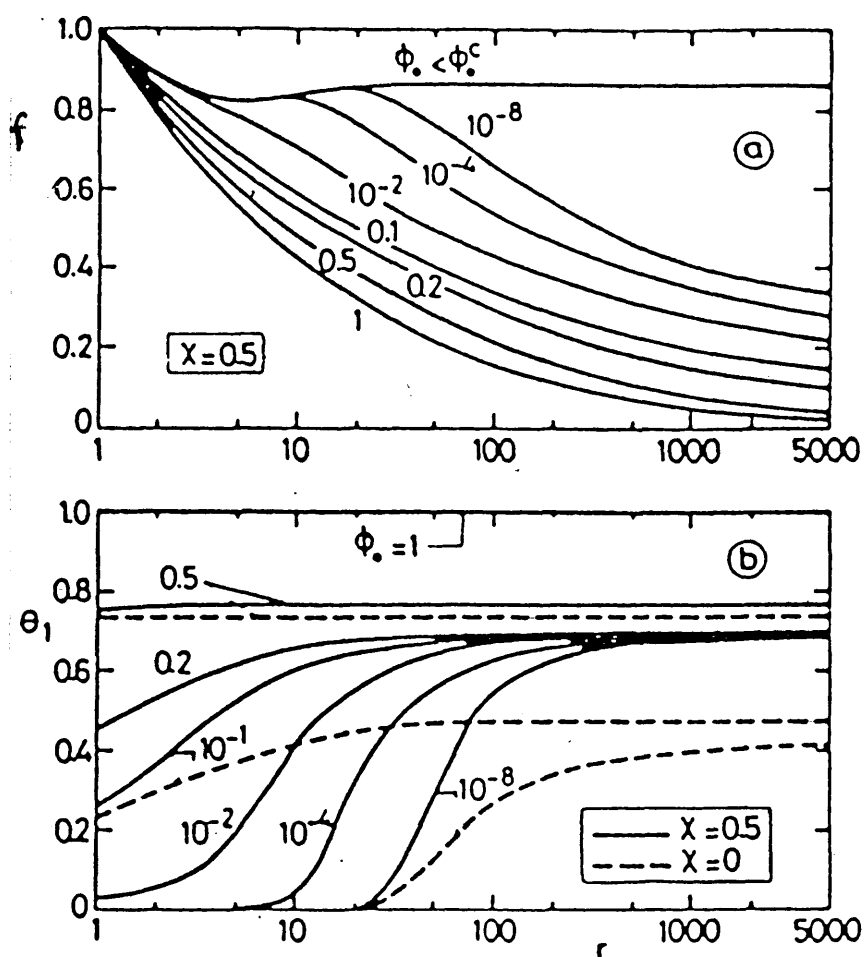


Fig. 4.23 Bound fraction and (a) direct surface coverable (b) as a function of chain length at various solution concentrations. Full curves $\chi = 0.5$; dashed curves $\chi = 0$; $\chi_s = 1$; hexagonal lattice ($\lambda = 0.5$) (Scheutjens & Fleer, 1982).

few basic interaction parameters). Scaling laws are concerned with global properties of polymers as they remain true for a large class of polymer chains. Flory (1941) has given an expression for the radius of gyration, S_g , of separate coils for a dilute solution in a good solvent

$$S_g = k a N_1^q \quad 4.21$$

where N_1 is the degree of polymerization and k is a constant which depends on the detailed monomer structure and on the solvent, a is the concentration of the monomer. Therefore in this relation q instead of k is universal. If the solvent is good q is close to $3/5$ but is equal to $1/2$ for a theta solvent. As discussed by De Gennes (1976, 1979), scaling laws make use of the exponent. Hence in Eq. 4.21 when the chain length is doubled in size S_g will be increased by a factor of 2^q .

De Gennes (1981) divided the adsorbed layer into three regions. These are shown in Fig. 4.24.

- a) A proximal region where the segment surface interactions are predominant, i.e. this is the train domain. Here the segment density depends on the adsorption energy;
- b) a central region where the chains are entangled. The segment density profile is universal and independent of bulk polymer concentration ϕ_* , and
- c) a distal region which is the outer region of the adsorbed layer and is controlled by a few large loops and tails. De Gennes assumed the concentration to relax exponentially towards the bulk solution.

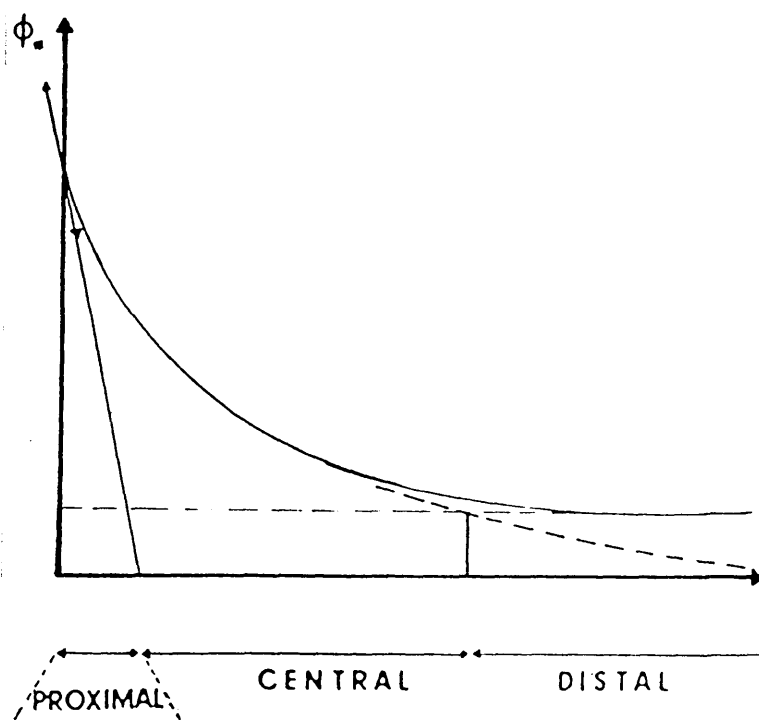


Fig. 4.24 Division of area in the proximity of a surface with polymers adsorbed according to scaling laws (De Gennes, 1981).

De Gennes (1981, 1982) also showed that the hydrodynamic thickness of the molecule t_h is controlled by the longest loops and tails and should be comparable to the radius of gyration. It is given by

$$t_h \sim k a N^{3/5} \quad 4.22$$

However, it is difficult to verify Eq. 4.22 experimentally as the measured value of t_h depends on the experimental technique used. Kato *et al.* (1981) from hydrodynamic measurements using photo correlation spectroscopy found the molecular weight dependence on t_h to be 0.56 for polystyrene lattices covered by polyethylene oxide in water while Cosgrove *et al.* (1984) using the same system obtained a higher exponent for $t_h \sim N^{0.8}$.

Scaling ideas also predict the structure of the adsorbed layer to be independent of bulk concentration as the free energy of the system is assumed to be dominated by the segments of the adsorbed layer. Scaling theory, however, does not specify the rising part of the isotherm. De Gennes (1983) also predicted the adsorbed amount Γ in the plateau to be independent of M.W. and Γ to be proportional to $\chi^{1/3}$. These interesting predictions are however difficult to test.

4.6 DISCUSSION

4.6.1 Comparison of experimental data with statistical theories

Though many theories have been put forward to explain the behaviour of polymers at interfaces, testing the predictions is difficult, since these theories are mainly for monodisperse systems and assume that the monomolecular films lie flat and unfolded at the interface, existing as a randomly coiled thread on the surface, loosely anchored to the aqueous substrate by polar groups distributed along the thread. However, even at low concentration the molecules of protein investigated here do not unfold completely at the interface. This is due to the existence of disulphide linkages which makes part of the molecule rigid. Therefore application of the random coil theory to protein films becomes ambiguous. Even for polymers these theories have not been verified quantitatively although attempts have been made to explain the experimental and the theoretical results qualitatively.

Comparison of the adsorption isotherm of Fig. 4.10 with those given by Scheutjens & Fleer (1982) in Fig. 4.21 indicates a similar general trend but quantitatively no comparison can be made as the

parameters observed from experiments carried out here are different from those required by the theoretical isotherms.

Comparison was also made between the experimental $\pi:A$ curves and the theoretical $\pi:A$ curves obtained by using Singer's equation for molecules adsorbing in all train configurations at an interface, for a_0 equal to 10 and 15 Å respectively. The general shape of the results at the air/water interface is found to be similar to those of Fig. 4.16 and lies between $Z = 2.1$ and $Z = 2.5$. This shows that the molecule is not in a fully expanded all train configuration and probably contains some condensed regions. This result may be due to loop formation, but comparison with Fig. 4.18 shows that although the areas occupied are now comparable the experimental curves are shallower.

An exact comparison is not possible since the proteins have a complex secondary structure which is not taken into consideration in the polymer adsorption theory, but would account for the proteins being in a more condensed state. Another point of importance is that the proteins are not homopolymers and the individual amino acids will occupy different areas when adsorbed at the surface. At the oil/water interface interactions with the adjacent medium complicate the situation and make comparison with theory even more difficult.

This is also seen when the pH of the solution is changed, the surface pressure attained for the same bulk concentration is different. This shows that the conformation of the adsorbed molecule is different at different pH values. Hence the number of amino acid residues in contact with the surface is also different for different values of pH.

4.6.2 Packing of macromolecules at interfaces

The conformation of macromolecules, especially proteins, at an interface requires detailed information about the composition of the molecules and the nature of the forces which exists between the sub-units. This detailed information is not often available, therefore a gap exists between the molecular properties of the macromolecules and the macroscopic surface properties that is not easy to bridge. Hence different measures are adopted to understand the physical properties of the macromolecular systems.

Feitjar *et al.* (1982) have used the soft particle concept for compact adsorbed macromolecules, this would mean that with increasing surface concentration, contraction may occur due to the forces exerted on a given molecule by surrounding molecules. He uses the equation put forward by Helfand *et al.* (1961) for the pressure of a two dimensional fluid of disk shaped or spherical hard particles which is

$$\pi = kT\Gamma/(1-\theta_s)^2 \quad 4.23$$

where k = Boltzman constant;

T = absolute temperature;

Γ = surface concentration in molecules per unit of surface area

θ_s = surface coverage.

and

$$\theta_s = a_m/a_o = \pi R_1^2 \Gamma \quad 4.24$$

where a_m = area of molecule;

a_o = area of the unit residue;

R_1 = the particle radius in the plane of the surface;

Γ = surface concentration.

According to Eq. 4.23 the slope of the π - Γ isotherm should increase steadily with increasing surface coverage. However, when the surface pressure is plotted against the surface concentration the resulting π - Γ curves are S-shaped (see Fig. 4.25). The slope of the plots decreases with increase in concentration. This decrease which occurs between 5 and 15 mNm⁻¹ has been explained by Feitjar *et al.* (1982). By changing the basic assumption that R remains constant, the π - Γ isotherm may become S-shaped. This shows that as the surface concentration increases the radius R of the macromolecules changes. This decrease may occur due to the forces exerted by the surrounding molecules causing the conformation to change.

The curves obtained in this study for serum albumin at both the air/water and oil/water interfaces are also S-shaped as shown in Figs. 4.26 and 4.27. The results show also the changes which occur as the pH is varied between 4 and 11. The surface coverage and the corresponding average molecular areas are obtained using Eq. 4.23 and are recorded in Table 4.1.

It can be seen that at high concentrations the area occupied by the molecules has been reduced to about half the value at low concentrations where there is no restriction on size. It is also seen that the radius of the molecules for a given surface concentration changes as the pH is varied. This is in part due to the conformational differences which occur in the bulk at different values of the pH and

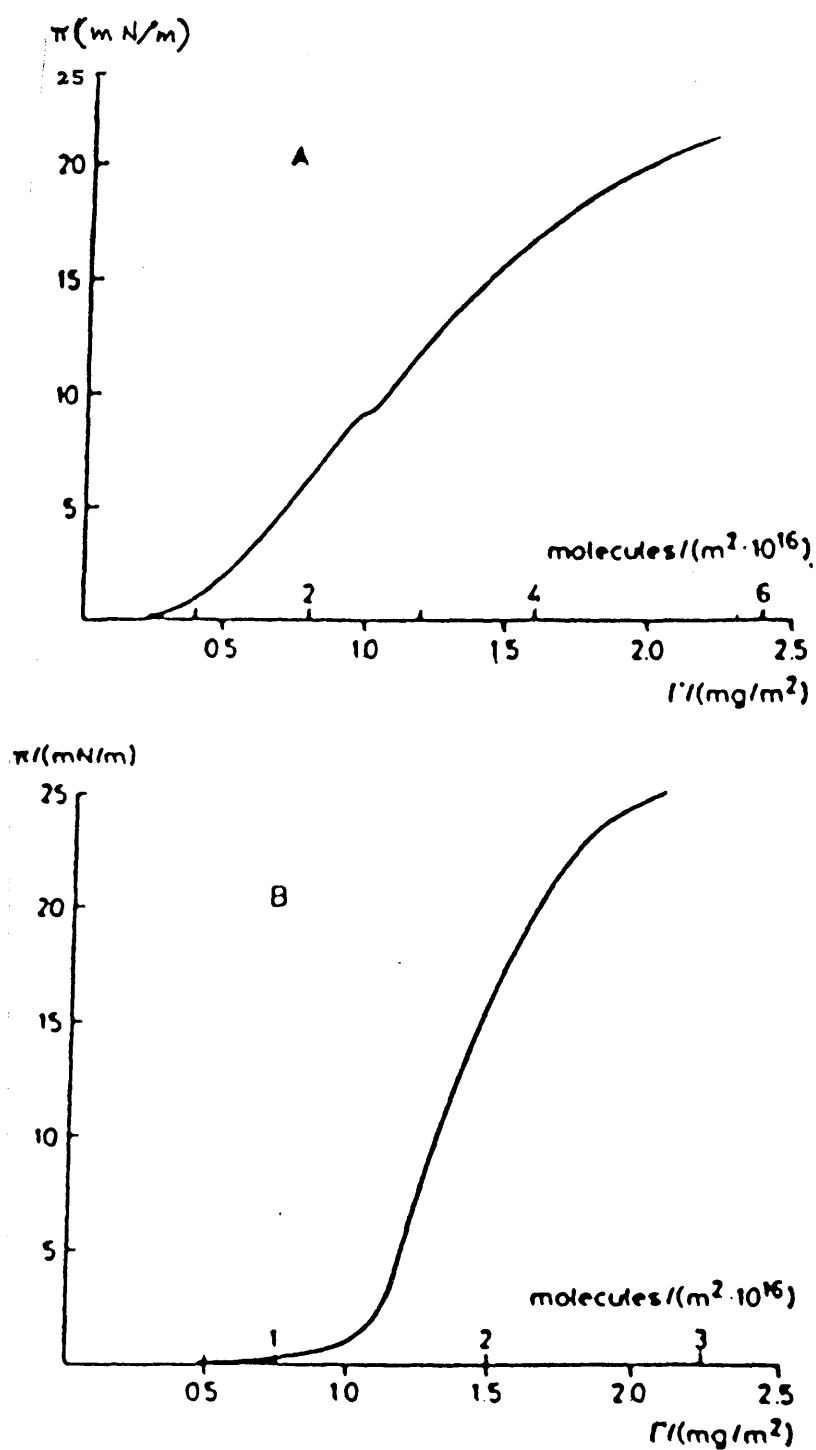


Fig. 4.25 Experimental π - Γ curves of proteins.
 (A) β -casein (MW=24,000); pH=7.0; $I=0.1$ eq/liter; $T=22^\circ\text{C}$.
 (B) Ovalbumin (MW=45,000); pH=6.7; $I=0.03$ eq/liter; $T=21^\circ\text{C}$.
 (Fertjar *et al.* 1982.)

TABLE 4.1

The surface coverage and the average molecular areas obtained for different values of pH at the air/water interface

pH 7.4

π mNm ⁻¹	Γ mgm ⁻²	Γ mol.m ⁻²	θ_s surface coverage (m ²)	Radius (nm)	Area (nm) ²
2	1.35	1.18 x 10 ¹⁶	.850	4.80	72.40
4	1.52	1.33 x 10 ¹⁶	.880	4.60	66.50
6	1.65	1.44 x 10 ¹⁶	.900	4.50	63.60
8	1.75	1.52 x 10 ¹⁶	.910	4.36	59.70
10	1.85	1.62 x 10 ¹⁶	.920	4.23	56.20
12	1.92	1.68 x 10 ¹⁶	.924	4.19	55.20
14	2.05	1.79 x 10 ¹⁶	.928	4.06	51.80
16	2.20	1.92 x 10 ¹⁶	.930	3.93	48.50
17	2.30	2.00 x 10 ¹⁶	.930	3.84	46.32

pH 4.7

2	1.40	1.22 x 10 ¹⁶	.840	4.68	68.81
4	1.65	1.44 x 10 ¹⁶	.880	4.40	60.82
6	1.80	1.57 x 10 ¹⁶	.897	4.26	57.01
8	1.92	1.68 x 10 ¹⁶	.907	4.15	54.11
12	2.10	1.83 x 10 ¹⁶	.921	3.99	50.01
18	2.50	2.18 x 10 ¹⁶	.930	3.68	42.54
20	2.75	2.4 x 10 ¹⁶	.930	3.51	38.70

pH 11

2	1.25	1.09 x 10 ¹⁶	.850	4.98	77.91
4	1.42	1.24 x 10 ¹⁶	.898	4.80	72.38
6	1.52	1.32 x 10 ¹⁶	.901	4.66	68.22
8	1.62	1.41 x 10 ¹⁶	.913	4.54	64.75
12	1.78	1.55 x 10 ¹⁶	.926	4.36	59.72
14	1.85	1.62 x 10 ¹⁶	.937	4.29	57.82
15	1.90	1.66 x 10 ¹⁶	.933	4.23	56.23

partly due to the interaction of the molecules with the adjacent phase.

From Fig. 4.26 (π vs. Γ) it is seen that for the same surface concentration, the surface pressure at the air/liquid interface has a maximum value of pH11. This shows that the number of amino acid residues in contact with the surface is maximum at pH11 and in the order pH11 > pH7.4 > pH4.7. However, at the liquid/liquid interface it is seen from Fig. 4.27 (Γ vs. π at liquid/liquid) that for the same surface concentration the maximum surface pressure is attained at pH7.4 indicating that the number of amino acid residues in contact with the surface is maximum at pH7.4 and in the order pH7.4 > pH11 > pH4.7.

In solution, the molecule of the protein is most extended at pH values away from the isoelectric point where the molecule is in its most compact state. At the air/liquid interface, as adsorption proceeds, a molecule at pH11 has most of its amino acid residues in contact with the surface while at pH4.7 and 7.3, although changes in the configuration of the molecules occur, it has fewer amino acid residues at the surface as compared with pH11.

However, at the liquid/liquid interface the adsorbed molecule of protein at pH11, although in its extended state in the bulk solution, tends to be more compact at the interface. This means that it has fewer amino acid residues in contact with the surface as compared with pH7. This shows that at the liquid/liquid interface close-packed loop formation is initiated at pH11 by the bulk oil phase.

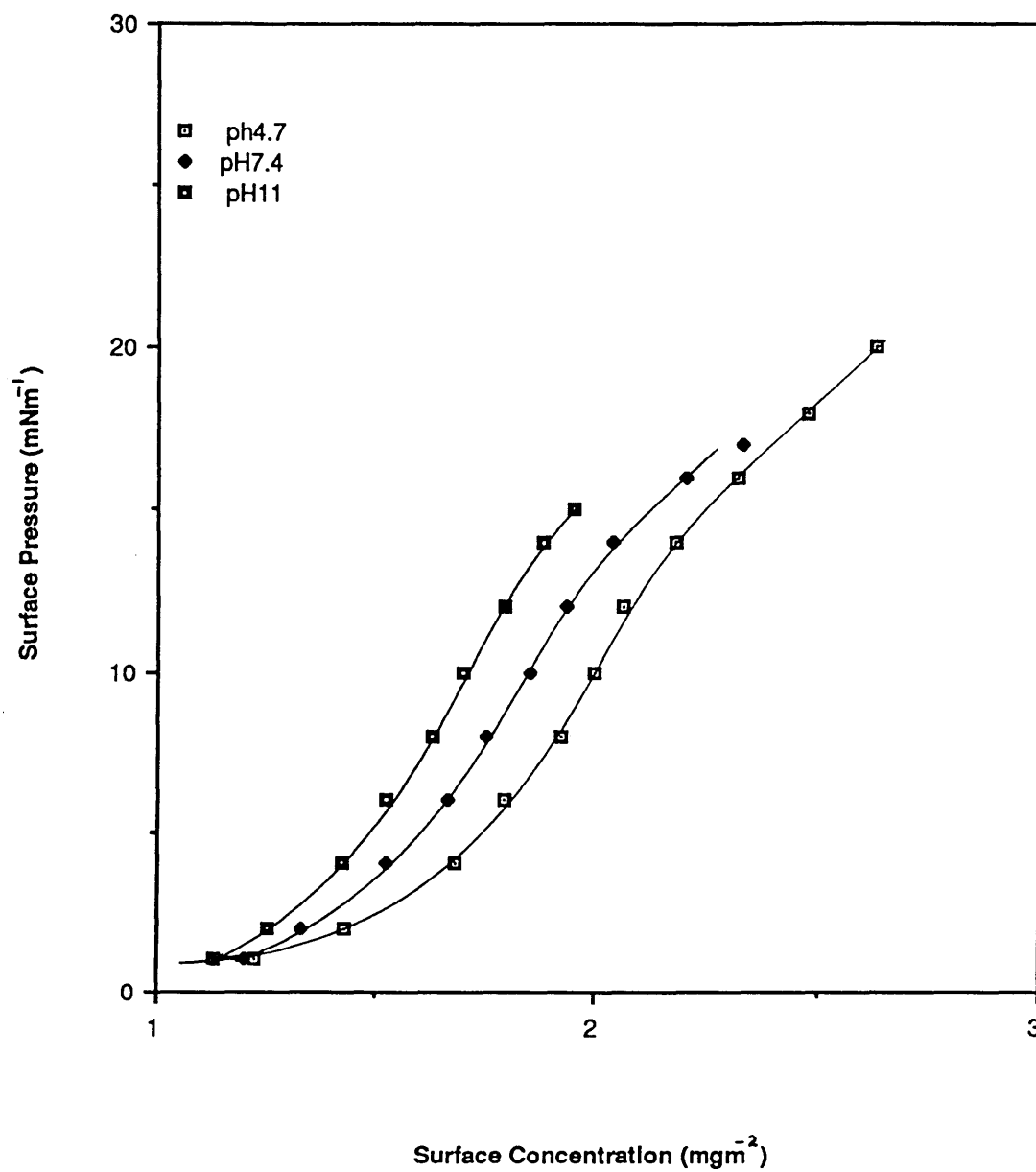


Fig. 4.26 Relative changes in surface pressure as the surface concentration increases for pH 4.7, 7.4 and 11 at the air/water interface. The slope of the curve decreases as the concentration increases for all the different pH values.

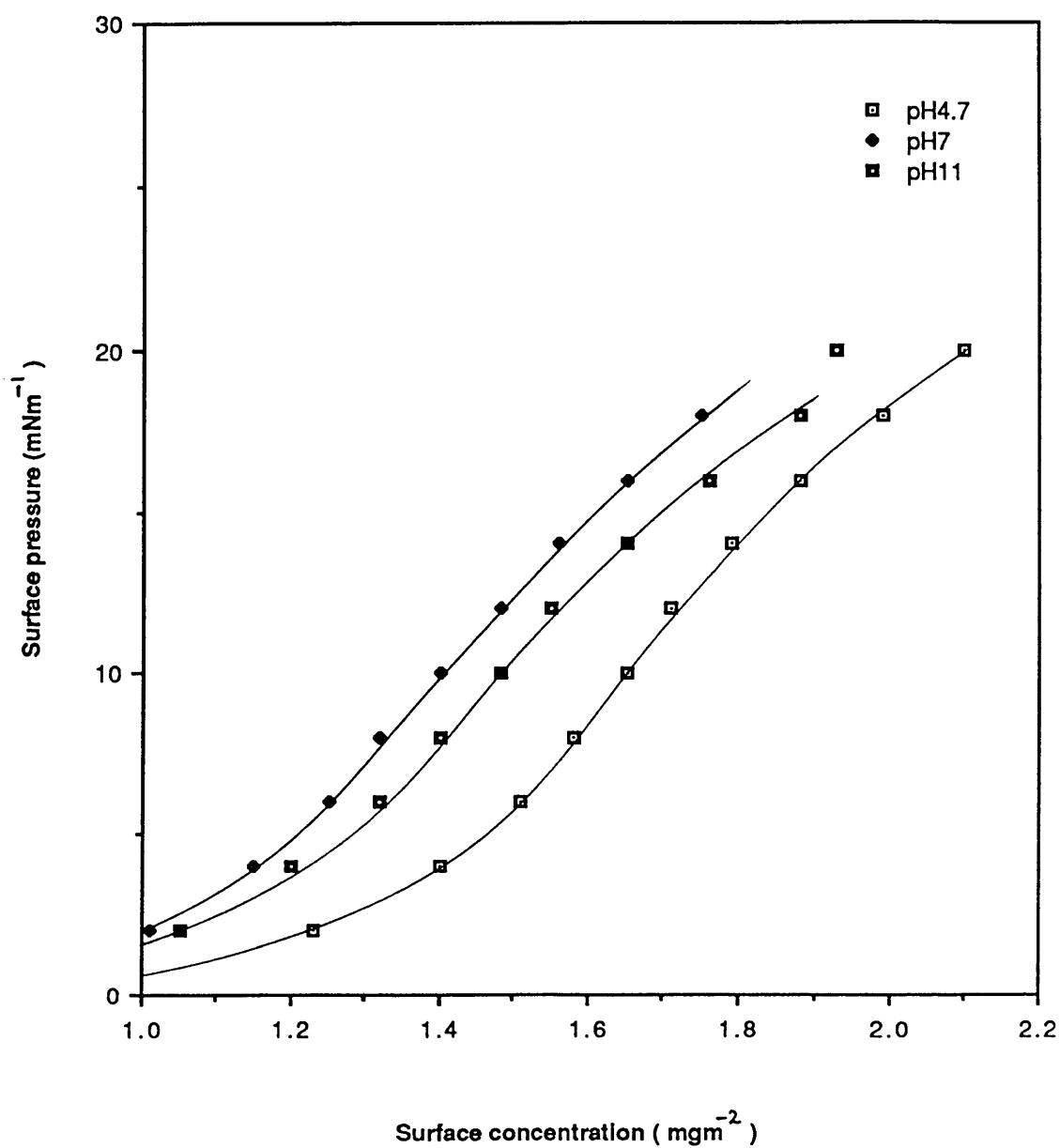


Fig. 4.27 Changes in the surface pressure values as the surface concentration increases at the oil/liquid interface for solutions at pH4.7, 7.4 and 11. Here also the slope decreases as the concentration increases.

4.7 CONCLUSIONS

In this chapter experimental results of interfacial concentration of HSA measured directly at both the air/liquid and liquid/liquid interfaces have been presented. These are further compared with values of interfacial concentration obtained indirectly by dynamic surface tension measurements at high frequency. Adsorption and surface pressure isotherms have also been presented for different values of pH at the air/liquid interface and compared with their equivalent isotherms at the liquid/liquid interface. It is seen that for all the different pH values π is higher at the liquid/liquid interface for equivalent surface values of the concentration. In all cases within the experimental range considered, the surface or interfacial pressures reach a limiting value but the surface or interfacial concentrations continue to increase slowly.

CHAPTER FIVE

DILATATIONAL RHEOLOGY

5.1 INTRODUCTION

As dynamic surface tension phenomenon are known to be sensitive to intermolecular interactions therefore an attempt has been made to understand these interactions by studying the dilatational rheology of Human serum albumin at the air/liquid and liquid/liquid interfaces. Monolayers adsorbed from bulk solution were used because, in experiments using spread monolayers, the presence of small organic molecules of the solvent used to promote spreading remain, even at low concentrations and are known to greatly change the rheological properties (Blank *et al.* 1970).

When proteins adsorb they form viscoelastic films. These films are physically strong and can withstand many different types of mechanical forces and hence are able to stabilize disperse systems such as foams and emulsions. Therefore, changes in the elasticity of protein film is of great technological importance since proteins form the basis of many industrial preparations such as are common in the food industries, pharmaceuticals, artists paints, cosmetics etc.

Rheology allows the prediction of the deformation resulting from the application of a given force system to a body. There are two ways in which the rheological properties of films adsorbed at interfaces may be studied.

In one, the response to forces causing dilatation or compression is studied. Here the shape of the film remains constant but the area is varied. At equilibrium the solutions of surface active substances

in the gaseous or liquid foams have a uniform surface coverage and the surface tension remains constant. As surface area is changed rapidly by expansion or compression, the surface tension will increase or decrease respectively and the surface will no longer be in equilibrium. In such a process the molecules will diffuse across the surface or adsorb or desorb to restore the surface tension to its equilibrium value. The response of the surface to dilatation or compression is described by a surface dilatational modulus, ϵ , which is given by

$$\epsilon = A \frac{d\gamma}{dA} \quad 5.1$$

where γ is the surface tension and A the area of the surface element considered. The magnitude of ϵ , in general, depends on the extent of the expansion or compression and the time scale applied in its measurements. The modulus is a complex function of the frequency and can be written as

$$\epsilon(i\omega) = \epsilon'(\omega) + i\epsilon''(\omega) \quad 5.2$$

where ω is the angular frequency.

ϵ' is the real part of the complex modulus. It is actually the elastic energy stored in the system and is also known as the dilatational elasticity, ϵ_d , i.e.,

$$\epsilon' = \epsilon_d \quad 5.3$$

ϵ'' is the imaginary part of the complex modulus and accounts for the energy dissipated in the system and can also be expressed in terms of the surface dilatational viscosity, η_d .

$$\epsilon'' = \omega \eta_d \quad 5.4$$

The second method by which rheological parameters can be studied involves shearing of the adsorbed film. The resistance of the surface due to a change in shape can be expressed by the surface shear modulus. This is measured by the surface shear elasticity, ϵ_s , and the surface shear viscosity, η_s . The detailed geometry of the apparatus used is important in the measurement of shear viscosity. The magnitude of the surface dilatational viscosity is found to be much larger than the surface shear viscosity for most systems particularly for those which show non-Newtonian behaviour (M. Van den Tempel, 1976). This is one of the reasons why in most applications dilatational properties are considered to be more important than the shear behaviour.

For insoluble monolayers the value for the dilatational modulus can be derived simply from the equilibrium relationship between the surface tension and the surface concentration, Γ , of the surface active material. It is obtained directly from the slope of the surface pressure vs. area plot.

$$\epsilon = - \frac{d\gamma}{d \ln \Gamma} \quad 5.5$$

During the deformation process if adsorption from the bulk to the surface is instantaneous then the surface dilatational viscosity will not exist indicating it is an elastic surface but if the diffusional interchange depends on the time scale of the deformation process then surface dilatational viscosity will be present. This delay in the

establishment of the local equilibrium at the surface indicates the presence of another rate dependent process, such as the existence of an adsorption barrier or the molecular rearrangement within the monolayer. The systematic study of the dilatational elasticity and viscosity shows that molecules in a monolayer exist in a series of physical states whose properties are highly dependent on the conformation that a molecule adopts on adsorption at an interface.

5.2 METHODS FOR DETERMINING DILATATIONAL/COMPRESSIONAL MODULUS

Three different methods have been developed to measure the real and imaginary parts of the complex modulus. They are:

1. Wave damping;
2. Surface tension response to sinusoidal area variation;
3. Stress relaxation.

5.2.1 Wave damping

Since ancient times there has been an interest in waves at liquid surfaces. This interest has led to the study of changes that occur at an interface due to wave motion.

Originally the damping of capillary waves of small wavelength or ripples were studied for the analysis of fundamental surface properties. It was Lord Kelvin (1871) who showed that the properties of ripples are mainly determined by surface tension, in contrast to the long waves where gravity is the main force. Hansen & Ahmed, (1971) Rillearts & Joos (1982), Van Voorst *et al.* (1964) and Luccassen *et al.* (1967) have made detailed studies of the properties of capillary waves.

Damping or loss of kinetic energy in capillary waves is mainly due to the viscous friction in the liquid below the surface, loss of such energy would therefore depend on the bulk viscosity of the liquid. During wave motion, as the surface undergoes alternate expansion and compression it causes local surface tension changes to take place if an adsorbed or spread monolayer of surface active material is present. This causes material transport along the surface and from the bulk to the surface. In such cases the pattern of liquid flow underneath the surface is altered, giving rise to a higher rate of energy dissipation by viscous friction and consequently to a higher damping than would be expected for the surface of a pure liquid. Initially analysis of the response of a surface to capillary waves or ripples was carried out for two extreme cases only. They were:

- i) the effect of ripples on the surfaces of pure liquids where there is no surface elasticity to influence the wave motion. This was done by Stokes (1847). He determined the effect of viscosity on the propagation of waves on the surface of pure liquids;
- ii) the effect of ripples on incompressible monolayers where the horizontal surface motion was virtually stopped. This was done by Lamb in 1945. It provided useful information about the properties of a liquid surface which determines the resistance against deformation, that is the time dependent properties of the adsorbed films such as film elasticity, relaxation processes etc. (Lucassen & Hansen, (1967); Lucassen (1968); Lucassen-Reynders *et al.* (1969); Garret *et al.* (1970).

In recent years it has become possible to analyse the behaviour of surfaces covered with viscoelastic films. A complete quantitative analysis by which the relationship between the dynamic surface properties and subsurface liquid flow due to ripples has been presented. The mathematical treatment for this phenomenon has been provided by Levich (1941); Hansen *et al.* (1964) Van den Tempel *et al.* (1965 and 1977).

The theory for the propagation of plane waves as developed by Van den Tempel *et al.* (1965) is briefly outlined below. The fundamental principles in the theory for the motion of the liquid on the surface involves two kinds of conservation equations. They are.

5.2.1.1 The conservation of mass:

$$\frac{dU}{dx} + \frac{dV}{dy} = 0 \quad 5.6$$

where U and V are the velocity component of x and y which are the horizontal and vertical coordinates of an element of the fluid.

5.2.1.2 The conservation of energy

This is represented by the Navier-Stokes equations which are:

$$\rho \frac{dU}{dt} + \rho U \frac{dU}{dx} + \rho V \frac{dU}{dy} = - \frac{dp}{dx} + \eta \Delta u \quad 5.7$$

and

$$\rho \frac{dV}{dt} + \rho U \frac{dV}{dx} + \rho V \frac{dV}{dy} = - \frac{dp}{dy} + \eta \Delta V - \rho g \quad 5.8$$

where ρ is the density, g the acceleration due to gravity, η the viscosity of the bulk and p the pressure gradient.

These equations must also satisfy two boundary conditions.

1. The vertical pressure component must be equal to the sum of the hydrostatic pressure of the gas phase and the Laplace pressure due to the curvature of the surface in the presence of the wave.
2. The horizontal component at the surface should be equal to the surface tension gradient with variation in area, $d\gamma/dA$.

To simplify these equations the only assumptions made by Van den Tempel were that the liquid is incompressible and the liquid layer has infinite depth, that is, the depth of the layer is large compared with the wavelength of the waves being considered. The variation in surface tension occurs due to periodic expansion and contraction of the surface. The solution of the equations is then given by:

$$U = U_1 \exp(i\omega t) + U_2 \exp(2i\omega t) + \dots, \quad 5.9$$

$$V = V_1 \exp(i\omega t) + V_2 \exp(2i\omega t) + \dots, \quad 5.10$$

These expressions are then substituted in the conservation equation, which along with their boundary conditions, provide a relationship defining the coefficients U_1 and V_1 . The solutions are obtained in terms of successive approximations.

The theory provides a relationship between the frequency (ω) and the wavelength ($\lambda = 2\pi/k$) by the following expression:

$$\omega^2 = g k + (\gamma k^3/\rho) \quad 5.11$$

where k = wave number;

g = acceleration due to gravity;

ρ = density of the liquid;

γ = surface tension.

and the magnitude of the damping coefficient as

$$\beta = 2\eta k^3 / 3\pi\rho\nu \quad 5.12$$

where η = viscosity

ν = frequency.

The hydrodynamic theory predicts a maximum in the damping coefficient. This has been demonstrated by Luccassen & Hansen (1966) for both spread and adsorbed monolayers, in systems where solute concentrations were sufficiently small that during wave motion diffusion from the bulk to the surface could be neglected. Luccassen & Hansen (1967) further extended these studies by measuring the damping of capillary waves on surfaces having appreciable diffusion interchanges.

They studied the effect of waves on surfaces covered with monolayers of fatty acids having chain lengths of C_5 - C_{10} . They found that the maximum values of the damping coefficients decreased with decreasing chain length from C_{10} - C_5 . This they attributed to the increasing effect of diffusion. They also observed that the peaks of the damping coefficients became progressively narrower as the chain length increased. This they suggested was due to increasing non-ideality of the higher fatty acids from the Szyszkowski and Langmuir surface equation of state.

The diffusion coefficient calculated for pentanoic acid and hexanoic acid agree well with the accepted values given by Longworth (1953). They are both approximately $5 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$. This suggests that for these acids there is no adsorption barrier. However for heptanoic acid and octanoic acid the calculated values of diffusion coefficient were smaller by a factor of about 10. From their investigations they have determined that a small error in damping coefficients (1% accidental, 1% systematic) causes a larger error in the calculated values of the diffusion coefficients. They also suggested that since these experiments were carried out near saturation adsorption this anomaly could also be due to an influence of relaxation effects within the surface film. They do not specify the type of relaxation process that would take place in a monolayer of fatty acids. It could be the rearrangement of the molecules or simply the collapse of the monolayer.

Hansen *et al.* (1968) studied the propagation of waves at the water/mercury and water/heptane interfaces. They pointed out that a liquid/liquid interface was much more difficult to study than an air/water interface. The wave numbers obtained experimentally were 0.7% greater than those predicted by the theory. The damping coefficient at the water/heptane interface was about 20% higher than the predicted value. A more serious discrepancy occurred at the water/mercury interface where the observed damping coefficient was over twice the predicted value. They attributed these differences to the contamination of the interface. The source of error could also be due to the difficulty in studying the waves at the liquid/ liquid interface.

While performing a capillary wave experiment Luccassen (1968) noted the existence of longitudinal surface waves. The damping

coefficient and the wave number of the longitudinal wave are determined by the elastic modulus rather than the surface tension values. Unlike capillary waves, the longitudinal waves are damped out much more rapidly except at high values of the elastic modulus. Such waves can be investigated experimentally over a wide range of frequencies so they are potentially of great interest in the study of monolayer relaxation phenomena. The results of Luccassen's experiments showed that the wavelength of the longitudinal waves was much greater than that of the capillary waves, by a factor of almost 30.

Luccassen studied the propagation of waves for many different mono-layers such as hexadecanol, methyl stearate, cholesterol, palmitic acid and lecithin. However, only mixed monolayers of cholesterol and lecithin were used for further quantitative studies as they gave reproducible values of wavelength and damping coefficient. For other monolayers, e.g. hexadecanol and palmitic acid, a quantitative study was not possible since the vibrations tended to induce monolayer collapse.

From the transverse wave characteristics it is possible in principle to determine the elastic modulus but only in the region of maximum damping. However, Luccassen found that the surface elastic modulus calculated from longitudinal wave data is more accurate than that obtained from capillary ripples. He made observations at both high frequency and low frequency where the system remained near equilibrium and a systematic difference of the elastic modulus was obtained from these measurements. He attributed this differences to neglect of the shear rheological parameters in the ripple theory or more likely to the relaxation phenomena occurring in the monolayer. However he did not relate this effect to any particular relaxation process.

5.2.2 Surface tension response to sinusoidal area variation

A convenient method for determining the surface dilatational/compressional modulus ϵ was then developed by Luccassen & Van den Tempel (1972). It involved the measurement of the surface tension variations that occur in the system in response to small sinusoidal area variations. In principle, the experimental conditions should be such that the periodic deformation is uniform over the entire area so that the measurements of variations of the surface tension values will provide all the information required for obtaining the surface dilatational modulus. For their experiments they used the Wilhelmy plate technique to measure the surface tension changes. The sample was held in a rectangular trough made of Teflon, having a movable barrier. The surface was periodically compressed and expanded by means of a small amplitude sinusoidal oscillation of the Teflon barrier at a frequency ν . A roughened glass plate which was suspended from an electrobalance was used as the surface tension sensor. It was connected to a chart recorder which recorded the changes in the surface tension values as a function of frequency. The recorder was equipped with an 'event marker' which gave a signal for maximum compression. If $\Delta\gamma$ is plotted against ΔA , an ellipse is usually drawn, since the tension and area changes are out of phase, as shown in Fig. 5.1. If the ellipse is boxed as shown in the figure, and if A, B, C and D are the points where the ellipse crosses the diagonals, then the phase difference θ is calculated using

$$\tan \theta/2 = \frac{AB}{CD} \quad 5.13$$

i.e.

$$\theta = 2 \tan^{-1} \left(\frac{AB}{CD} \right) \quad 5.14$$

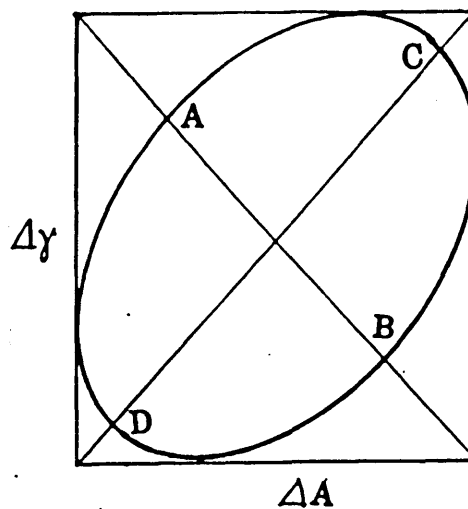


Fig. 5.1 Change in surface tension values plotted against change in area.

The dilatational modulus is then given by

$$\epsilon = \epsilon |e|^{i\theta} = |\epsilon| \cos \theta + i |\epsilon| \sin \theta = \epsilon' + i\epsilon'' \quad 5.15$$

where ϵ' = storage modulus;

ϵ'' = loss modulus;

The surface dilatational modulus can also be calculated by the following equation if the applied variations in area and surface tension are of sufficiently small amplitude.

$$\epsilon = \frac{L \Delta \gamma}{\zeta_B} = \frac{A d\gamma_{\max}}{dA_{\max}} \quad 5.16$$

where L = distance between the barrier and end of the trough;

ζ_B = amplitude of the oscillation of the barrier.

Luccassen & van den Tempel (1972) also derived relationships between the surface dilatational modulus, angular frequency and diffusion coefficients for diffusion controlled processes. Taking account of the phase lag the following equations were derived:

$$|\epsilon| = \frac{\epsilon_o}{\sqrt{1+2\xi+\xi^2}} \quad 5.17$$

where $\xi = \frac{dc}{d\Gamma} \sqrt{\frac{D}{2\omega}}$ 5.18

and $\epsilon_o = -\frac{d\gamma}{d\ln\Gamma}$ 5.19

and $\tan\theta = \frac{\xi}{(1+\xi)}$ 5.20

where c = bulk concentration;

D = diffusion coefficient;

Γ = surface concentration;

γ = surface tension.

They investigated surface films of decanoic acid. Values of $|\epsilon|$ were obtained from the measured surface tension variations by means of Eqn. 5.16.

They found good agreement between experimentally obtained and the predicted values. Their results are shown in Figs. 5.2 and 5.3. They also calculated the adsorption isotherm from the dynamic measurements and found it to be in good agreement with the observations.

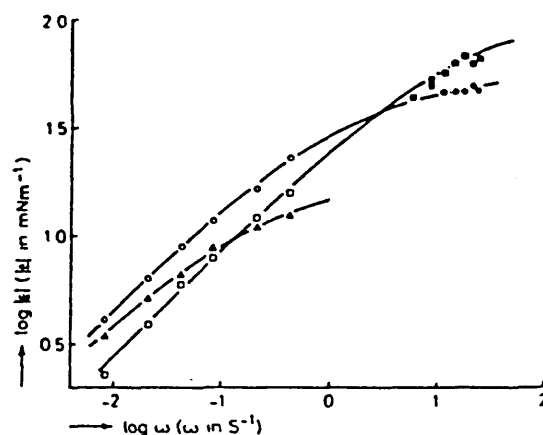


Fig. 5.2 Absolute value of surface dilatational modulus for decanoic acid solutions. $\circ$ $2.0 \times 10^{-8} \text{ mol cm}^{-3}$; $\square$ $10^{-7} \text{ mol cm}^{-3}$; Δ $4.5 \times 10^{-8} \text{ mol cm}^{-3}$; $\bullet$ $4.5 \times 10^{-8} \text{ mol cm}^{-3}$. Drawn lines represent theory. (Lucassen *et al.* 1972.)

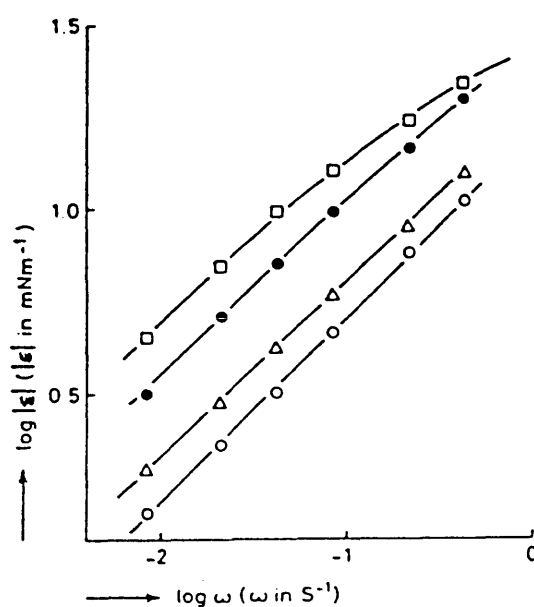


Fig. 5.3 Absolute value of surface dilatational modulus for decanoic solutions. $\bullet$ $3.0 \times 10^{-8} \text{ mol cm}^{-3}$; Δ $1.5 \times 10^{-7} \text{ mol cm}^{-3}$; $\circ$ $7.0 \times 10^{-8} \text{ mol cm}^{-3}$; $\square$ $2.0 \times 10^{-7} \text{ mol cm}^{-3}$. Drawn lines represent theory. (Lucassen *et al.* 1972.)

Although surface tension changes caused by local, purely periodic area variations produced a simple method for determining the dilational modulus, Luccassen *et al.* (1972) demonstrated that the range of frequencies that could be used was very small. The wavelength could not be much smaller than the length of the trough due to the very strong damping of the longitudinal waves. This puts an upper limit on the frequency. The fraction of the wave amplitude which remains after the wave has travelled one wavelength becomes very small and hence difficult to detect.

A lower limit to the frequency range is established by the condition that the wavelength should not exceed the length of the trough too much in order to avoid the influence of reflections from the wall opposite the moving barrier. Hence a detailed analysis of the results becomes restricted due to the limited frequency range that is available.

Luccassen & Barnes (1972) also pointed out that the extent of deformation resulting from expansion and compression of a surface should be sufficiently homogeneous over the whole enclosed area so as to permit accurate measurements. They showed that the possible sources of experimental errors in general have a relatively larger effect on phase angle measurements than they have on measurements of $|\epsilon|$. The wave propagation number W which is a dimensionless quantity is given by:

$$W = \frac{|\epsilon|^{\frac{1}{2}}}{L\omega^{\frac{3}{4}}\eta^{\frac{1}{4}}\rho^{\frac{1}{4}}\sin(\frac{\pi}{8} + \frac{\theta}{2})} \quad 5.21$$

where L = the distance between the oscillating barriers;

ω = angular frequency;

η = liquid viscosity;

ρ = liquid density.

They showed that if W tends to be larger than 4 the error in θ (phase angle) is less than 2° and that in $|\epsilon|$ is less than 1%. They have further demonstrated that the quantity L does not strictly refer to the distance between the barriers but is a characteristic dimension of a uniform surface which can undergo a compression/expansion type of deformation.

On the basis of this Luccassen & Giles (1975) studied the properties of non-ionic surfactants at various frequencies. The schematic representation of their experimental set up is given in Figs. 5.4a and 5.4b.

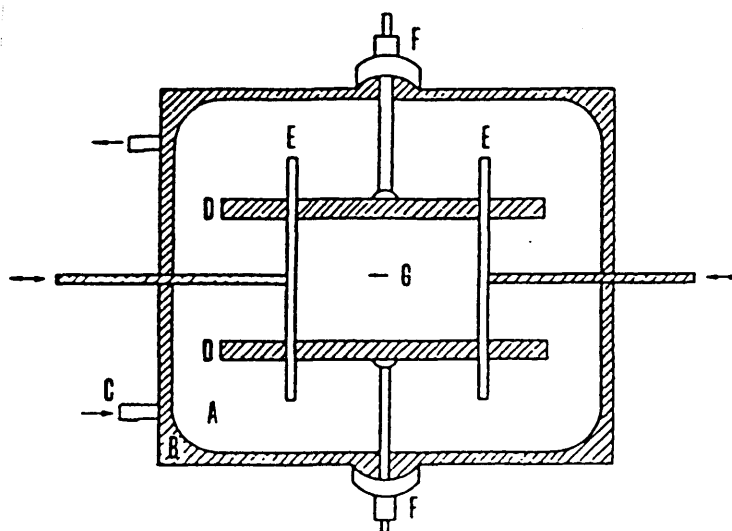


Fig. 5.4a Trough for dynamic surface measurements: A, stainless steel dish; B, aluminium mantle; C, inlet thermostating water; D, lower PTFE bars; E, oscillating bars; F, attachment lower bars; G, Wilhelmy plate. (Lucassen & Giles, 1975.)

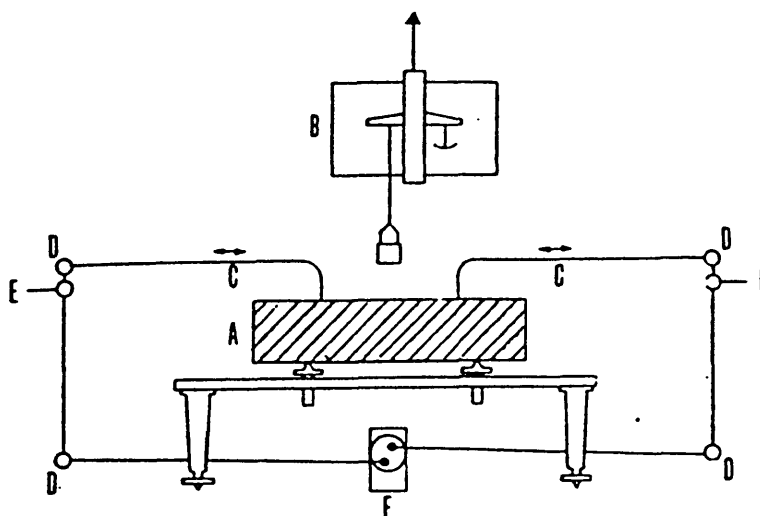


Fig. 5.4b Measuring equipment for surface dilatational modulus (side view): A, trough and mantle; B, electro-microbalance and Wilhelmy plate; C, oscillating barriers; D, knee joints; E, pivots; F, motor. (Lucassen & Giles, 1975.)

The surface to be compressed and expanded was enclosed between four PTFE barriers, two fixed and two movable. The surface was periodically compressed and expanded by means of the sinusoidal oscillation of these two movable barriers. The variation in surface tension was measured using the Wilhelmy plate technique. They studied non-ionic surfactants both above and below the critical micellar concentration (c.m.c.). The behaviour below the c.m.c was found to be diffusion controlled and no evidence for the existence of a barrier to adsorption and desorption was observed. On comparing their results with experiments carried out by F. van Voorst Vader *et al.* (1964) in continuous surface expansion of solutions, they concluded that there is a very close correlation between the oscillatory and steady state dynamic surface properties in the diffusion dominated region.

Above the c.m.c., however, they observed a rapid decrease in elasticity with increase in concentration accompanied by an increase in the phase angle to values in excess of $\pi/4$. They attributed this behaviour to the existence of micelles, and explained it on the basis that any surface deformation will cause a change in subsurface monomer concentration. Consequently the monomer micellar equilibrium will be disturbed. The way in which the system responds to this will depend on the micellar relaxation time, i.e. the rate at which micelles form and break up in solution.

5.2.3 Stress relaxation

In stress relaxation experiments, the surface area is subjected to a sudden change after which the surface tension relaxation is measured. The time scales of the relaxation processes can then be determined from the relaxation curves.

It is essential that viscoelastic parameters are obtained after equilibrium has been attained, therefore it would be extremely time-consuming to use either the wave damping or oscillation method as the protein solution used required a period of over 16 hours in order to reach a steady state. Hence for systems containing large molecules like proteins which, because they are complex in structure also have slow relaxation processes, the stress relaxation method appears to be the most suitable.

Veer *et al.* (1973) carried out a detailed investigation of the relaxation processes at a surface covered with a surfactant monolayer and solid surfactant particles which represents the condition after monolayer collapse. They used stress relaxation experiments along

with longitudinal surface waves, and gave a quantitative interpretation of the surface tension relaxation phenomena in terms of two processes.

- (i) Surfactant exchange between the monolayer and the underlying solution by means of bulk diffusion.
- (ii) Surfactant exchange between the monolayer and the particles on the surface.

The second process is a combination of two different processes, firstly the surface diffusion to and from the particle governed by the surface concentration gradient and secondly a rate process at the surface of the particle. Veer *et al.* suggested that the latter of these two processes will be the rate determining step at the air/liquid interface. They carried out measurements on an aqueous suspension containing dodecanol, tetradecanol and hexadecanol. The suspensions were prepared by dissolving the surfactants in water at a temperature well above their melting points and then cooling to room temperature.

The dilatational modulus and the phase angle calculated from the longitudinal wave experiments were found to be in good agreement with the theoretical values. From the analysis of the longitudinal wave results the surface tension relaxation curves were predicted and compared with the experimental values obtained by performing stress relaxation experiments. The predicted values were found to be in good agreement with the experimental curves.

Stress relaxation experiments were also done by Loglio, Tesli & Cini (1977) using a different method. They determined the surface dilatational modulus as a function of frequency, by the Fourier trans-

formation of the surface tension relaxation function $\Delta\gamma(t)$ obtained from a stress relaxation experiment. The transformation from time to frequency was accomplished by the following relationship:

$$\epsilon = \frac{\text{Fourier transform of } \Delta\gamma(t)}{\text{Fourier transform of } (\Delta A(t)/A)} \quad 5.22$$

The Fourier transform of a function $f(t)$ is defined by

$$\text{F.T.}[f(t)] = \int_{-\infty}^{\infty} f(t)e^{-i\omega t} dt \quad 5.23$$

where ω is the angular frequency.

Since for the above condition $f(t) = 1$ for $t < 0$, Eq. 5.23 reduces to

$$\text{F.T. } (f(t)) = \int_0^{\infty} f(t)e^{-i\omega t} dt \quad 5.24$$

Since $\Delta A(t)/A$ is a step function, i.e. $\Delta A(t)/A = 0$ for $t < 0$ and $\Delta A(t)/A = \text{constant}$ for $t \geq 0$, the Fourier transformation was obtained analytically. Hence it was possible to obtain numerically the Fourier transformation of the surface tension decay, $\Delta\gamma(t)$.

The experimental set-up involved a Langmuir trough with two movable barriers. By means of a Wilhelmy plate, the rapid changes in surface tension values following a sudden change in area were continuously recorded. The surface tension relaxation curve thus obtained is shown in Fig. 5.5.

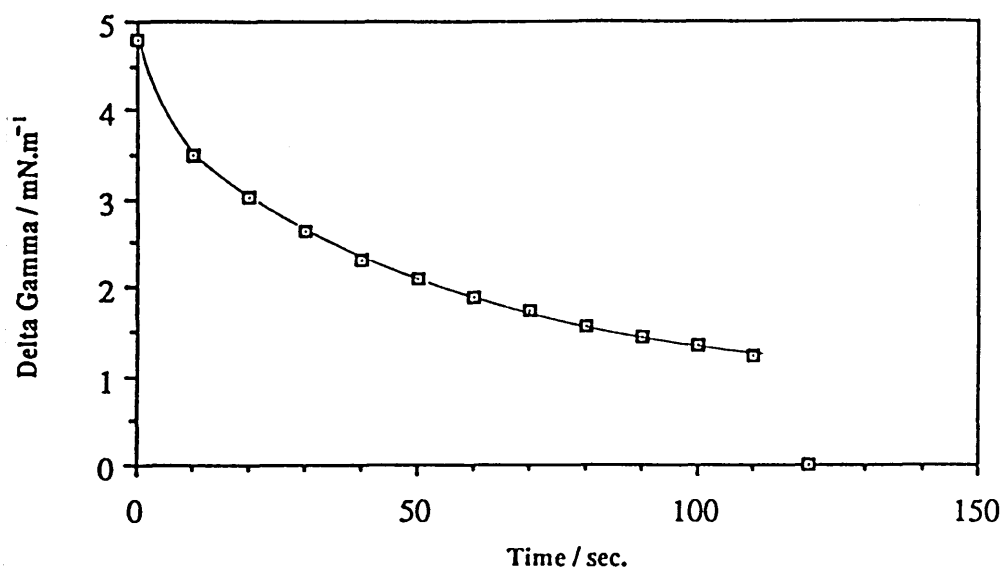


Fig. 5.5 Dynamic surface tension data for dimethyldodecylphosphine oxide, obtained by stress relaxation experiment (Loglio *et al.* 1977).

They studied three different surfactants, dimethyldodecylphosphine oxide, sodium dioctylsulphosuccinate and heptaethylene glycol nonylphenyl ether.

For the numerical calculation of the Fourier transformation, data had to be recorded at regular intervals of three seconds. The values of the modulus obtained in this way as a function of frequency were found to be comparable with those published by Luccassen & Giles (1975). They were also able to calculate the modulus at a very low frequency, 1×10^{-4} Hz, a range which was not possible by the use of the longitudinal wave method.

The accuracy of the numerical analysis was further improved by Loglio *et al.* (1979) by reducing the time interval of data sampling from 3 secs. to 0.1 sec. This technique has also been used by Callaghan *et al.* (1981) for measuring the dilatational properties of crude oil.

5.3 DILATATIONAL VISCOSITY

Another rheological parameter which measures the resistance against change in area is the surface dilatational viscosity (η_d). For a number of insoluble monolayers the surface behaves as a purely elastic body where the surface dilatational modulus is independent of the wave frequency. Hence the elasticity modulus $\epsilon = \epsilon_d(i\omega)$, with surface dilatational viscosity, η_d equal to zero. For a viscoelastic body the diffusional interchange during surface expansion and compression is dependent on the frequency. As a result the surface dilatational modulus is also frequency dependent and the surface dilatational viscosity will no longer vanish.

During a surface dilatation as for instance, during wave motion, the surface tension is not instantaneously adjusted. Then another rate dependent process, such as molecular rearrangements within the monolayer must exist. Very little experimental work involving surface dilatational viscosity has been published. The concept was introduced by Bousinnesq (1913). He defined it by the following expression:

$$\Delta\gamma = \eta_d \frac{dA}{A dt} \quad 5.25$$

where $\Delta\gamma$ = change in surface tension;

η_d = dilatational viscosity;

A = area of the surface;

t = time.

It was nearly half a century later that the first reliable measurements were carried out by Van voorst Vader, Erken & Van den Tempel (1964). Their method was based on the relationship described by Boussinesq. Their apparatus consisted of a Langmuir trough with two symmetrical motor driven barriers. The velocity of these barriers was chosen such that the relative rate of the surface extension, $d\ln A/dt$, was kept constant. The increase in the surface tension due to an expansion was measured using the Wilhelmy plate technique. The ratio of the steady state surface tension excess, $\Delta\gamma$, and a relative extension rate, enabled the dilatational viscosity to be determined. They further derived a relation between $\Delta\gamma$ ($d\ln A/dt$) and the bulk diffusion coefficient, D, by using the Szyszkowski, Langmuir & Gibbs adsorption equations. This relationship is given by:

$$\Delta\gamma = RT\Gamma_{\infty} \ln \left\{ \frac{2(1+q)}{(1+q-m) + \sqrt{(1-q+N)^2 + 4q}} \right\} \quad 5.26$$

where $q = \frac{C_b}{a_s}$ and $m = \frac{\Gamma_{\infty}}{a_s} \sqrt{\frac{\pi\phi}{2D}}$

and C_b = bulk concentration;

a_s = Szyszkowski constant;

ϕ = $d\ln A/dt$;

Γ_{∞} = saturation adsorption;

R = gas constant;

T = absolute temperature.

They studied solutions of lauryl alcohol-5 ethylene oxide and dodecanediol-1,2. They evaluated the diffusion coefficients for these solutions using the above relationship and found them to be $1.2 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ and $0.77 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ respectively. When compared with those predicted using empirical equations by Longworth (1953) such as:

$$D \times 10^6 = 24.182 / (V_m^{1/3} - 1.280) \quad 5.27$$

where V_m is the molecular volume, they found the experimental diffusion coefficients to be 1.5 to 2 times larger. The differences were attributed to possible mechanical defects of the apparatus, such as leakage along the barriers or uneven expansion of the surface which could lead to a non-uniform surface tension excess along the surface. However, they have mentioned in their article that they verified experimentally the evenness of the expansion. Also, if a Langmuir trough is properly constructed, leakages can be avoided. Therefore, it is possible that this discrepancy is true and not due to any error. Nevertheless they concluded that there is no appreciable energy barrier against adsorption. They also found that agreement between the theory, expressed in Eq. 5.26 and their experimental values was excellent. This is shown in Fig. 5.6.

Rillearts & Joos (1982) developed a new technique for measuring surface dilatational viscosity. The dilatation of the surface was achieved by applying a velocity gradient by means of a strip drawn through the surface, as shown by the schematic diagram below (Fig. 5.7).

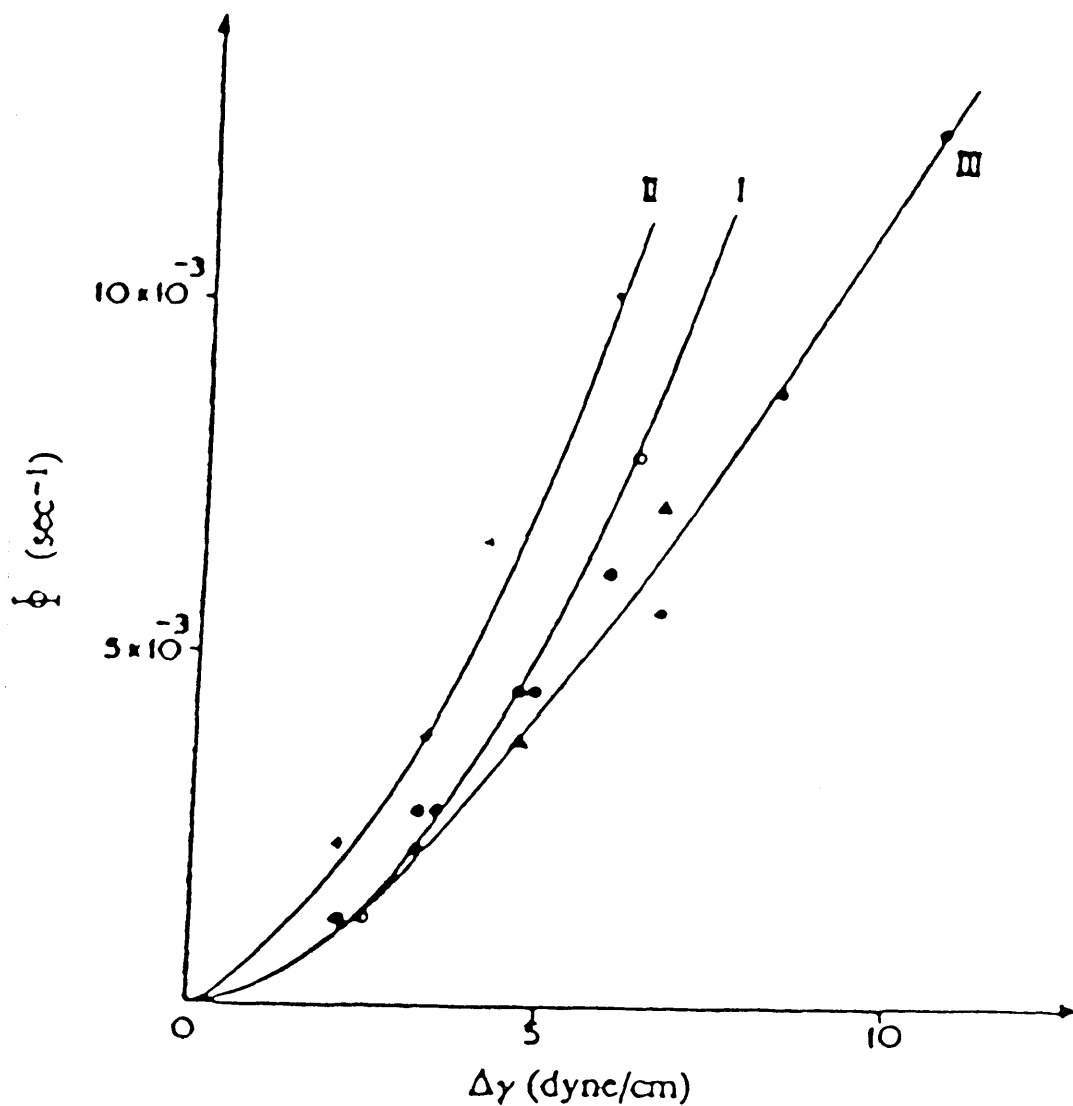


Fig. 5.6 Relative rate of surface tension ϕ against surface tension deviation $\Delta\gamma$. o, dodecanediol-1,2; $C_b = 466 \times 10^{-8}$ mole/cm³; curve I, theoretical values assuming $D = 1.2 \times 10^{-5}$ cm²/sec. + : lauryl alcohol-5 EO., $C_b = 2.62 \times 10^{-8}$ mole/cm³; curve II, theoretical values assuming $D = 0.77 \times 10^{-5}$ cm²/sec. o: the same, $C_b = 1.90 \times 10^{-8}$ mole/cm³; curve III, theoretical values assuming $D = 0.77 \times 10^{-5}$ cm²/sec. Δ : the same, $C_b = 1.85 \times 10^{-8}$ mole/cm³. (van Voorst Vader *et al.* 1964.)

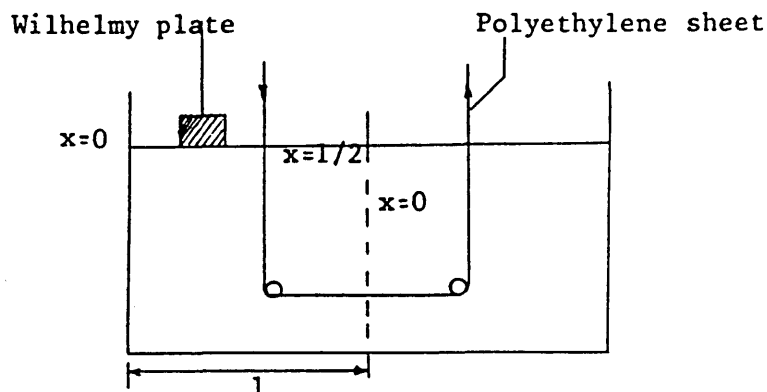


Fig. 5.7 Schematic diagram of the apparatus used by Rillearts & Joos (1982).

They investigated solutions of Na-myristate and Na-laurate. The analysis of their data was based on the theory of Van Voorst Vader *et al.* (1964). They found that their results agree well with the theory. The diffusion coefficients for these surfactants were found to be of the order of $3 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$. They did not however compare their results with those predicted using the empirical equation of Longworth. They further developed a theory for mixed surfactants and found that agreement between their experimental data and that predicted by their theory was reasonable (see Fig. 5.8). Using this technique they were also able to achieve a much higher dilatation rate than Van Voorst Vader *et al.*, (1964) i.e. of up to 1 unit of area per sec.

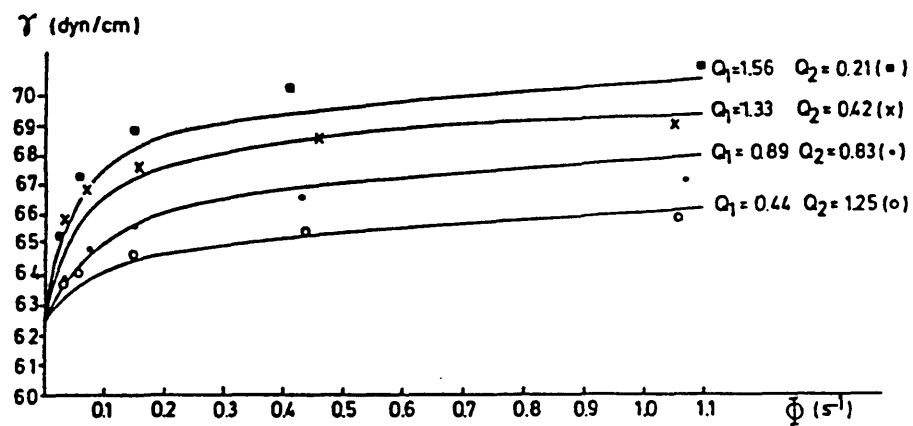


Fig. 5.8 Dynamic surface tension of Na-laurate + Na-myristate solutions $Q_1 = C_1/a_1$ (for myristate, $Q_2 = C_2/a_2$ (laurate)). Solid lines calculated. (Rillearts *et al.* 1982)

L. Ting *et al.* (1984) studied the dynamic interfacial properties of octanoic acid using longitudinal waves. For such waves the response of the surface is dependent on a combination of the surface shear viscosity, η_s , and the surface dilatational viscosity, η_d . In order to differentiate between them, they determined the surface shear viscosity in a separate experiment using a deep channel viscous traction viscometer. The results show that the surface dilatational viscosity is approximately two orders of magnitude greater than the surface shear viscosity. Their results are shown in Fig. 5.9. These results are in good agreement with those observed by Maru & Wasan (1979) and by Djabbarah & Wasan (1982) for other systems.

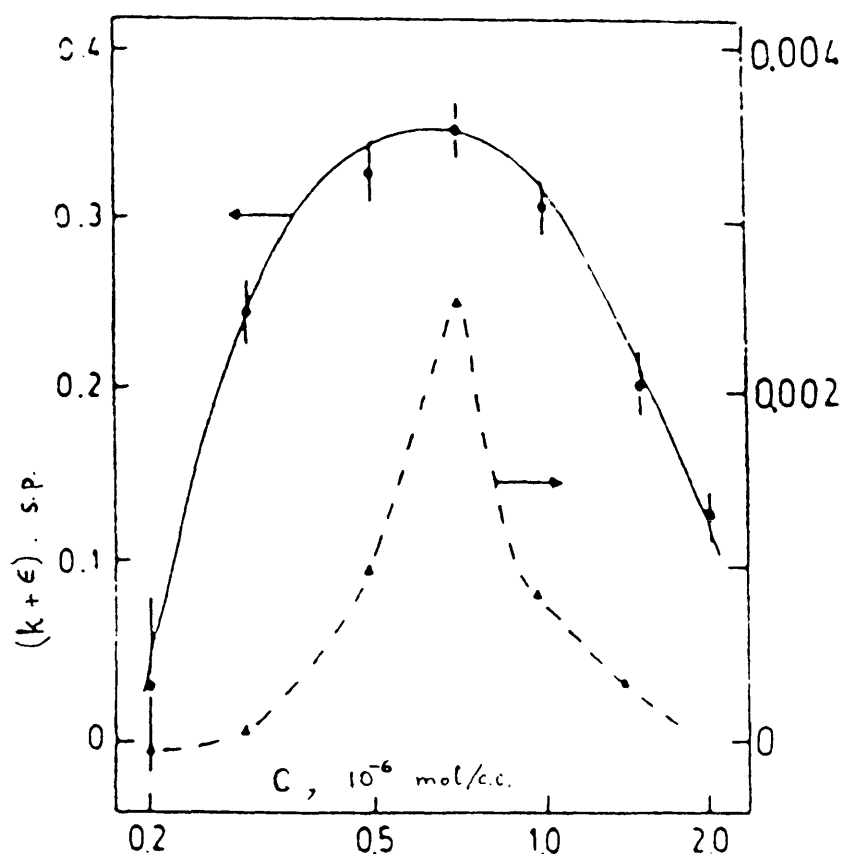


Fig. 5.9 The surface viscosities at different concentrations. The solid line represents the sum of the two viscosities; the dashed line the surface shear viscosity (Ting *et al.* (1984))

The experiments indicate that while the surface dilatational viscosity has only a minor effect on the dilatational modulus the contribution due to surface shear viscosity is negligible.

5.4 CHOICE OF EXPERIMENTAL METHOD

From the numerous techniques described for the evaluation of the surface dilatational/compressional rheological parameters, the stress relaxation method used by Loglio *et al.* (1977, 1979) was considered to be most suitable for adsorbed films of proteins which take a long time to reach equilibrium.

The advantages of this technique are:

1. Determination of the entire spectrum of the viscoelastic parameters by the method of damping of the waves at the interface would require observations to be made over a wide range of frequencies at both the air/liquid and liquid/liquid interfaces for a given concentration. However, in the stress relaxation method a single experiment would be sufficient for investigating the entire range.
2. As viscoelastic parameters are obtained after a system has reached equilibrium, it would be extremely time consuming to use either the wave damping or the oscillation method for systems such as proteins which take a long time to reach a steady state.
3. Measurements of the wavelength and the damping coefficient using the capillary waves method are subject to large errors at liquid/liquid interfaces when compared with observation made at air/liquid interfaces. This arises as a result of difficulties in the alignment of the oscillator and receiver.

4. The stress relaxation method enables the data to be obtained at very low frequencies, viz. lower than 10^{-4} Hz which is not possible by using either the wave damping or the oscillation method.

5. Sampling of data at intervals of 0.1 seconds over the long times required to reach equilibrium would be very tedious and would make data handling difficult.

Preliminary trials showed that the decay was not a simple exponential function of time, therefore an analytical expression determined by a curve fitting procedure was used to represent the decay function. The Fourier transforms of the analytical expression were then easily obtained.

5.4.1 Curve Fitting procedure

In an ideal case, when the decay is due to only one relaxation mechanism the decay curve can be represented by an exponential equation of the type:

$$\Delta\gamma(t) = \alpha e^{-\beta t} \quad 5.28$$

where β is the characteristic frequency of the relaxation process.

In a practical system a range of independent relaxation mechanisms occur simultaneously, each with its own characteristic frequency, ω . Thus the most probable equation to fit this decay curve would be a summation of exponential functions, and is given by:

$$\Delta\gamma(t) = \sum_{k=1}^n \alpha_k e^{-\beta_k t} \quad 5.29$$

where β_k is the characteristic frequency of the k th process and $\alpha_k/\Sigma\alpha_k$ is the fractional contribution of that process.

Estimates of α_k and β_k are obtained from observations of $\Delta\gamma(t)$. Errors involved in measurements of times are assumed to be negligible. If $\Delta\gamma_j$ is the j th observed value, then the residual w_j is given by:

$$w_j = \Delta\gamma_j - (\Sigma\alpha_k e^{-\beta_k t})_j \quad 5.30$$

The least-squares technique cannot be applied to the above equation because of its non-linearity with respect to β_k . Thus it is necessary to linearise the function and this is achieved by expanding the term $\alpha_k e^{-\beta_k t}$ (Guest, 1961). Thus:

$$(\Sigma\alpha_k e^{-\beta_k t})_j = (\Sigma\bar{\alpha}_k e^{-\bar{\beta}_k t})_j + \Sigma\left(\frac{\partial F}{\partial \alpha_k}\right)_j \alpha'_k + \Sigma\left(\frac{\partial F}{\partial \beta_k}\right)_j \beta'_k \quad 5.31$$

where

$$\alpha_k = \bar{\alpha}_k + \alpha'_k$$

$$\beta_k = \bar{\beta}_k + \beta'_k$$

and

$$F = \alpha_k e^{-\beta_k t}$$

$\bar{\alpha}_k$ and $\bar{\beta}_k$ are first approximations to the value of α_k and β_k .

Then if

$$\Delta\gamma'_j = \Delta\gamma_j - (\Sigma\alpha_k e^{-\beta_k t})_j \quad 5.32$$

$$\bar{x} = \frac{\partial \bar{F}}{\partial \bar{\alpha}_k} \text{ and } \bar{y} = \frac{\partial \bar{F}}{\partial \bar{\beta}_k} \quad 5.33$$

where

$$F = \alpha_k e^{-\beta_k t}$$

Thus Eq. 5.30 becomes:

$$w_j = \Delta\gamma_j' - (\Sigma\alpha_k'\bar{x} + \Sigma\beta_k'\bar{y}) \quad 5.34$$

The least-squares principle states that the parameters are to be chosen to minimize Σw_j^2 , thus leading to the following set of equations:

$$\frac{\partial \Sigma w_j^2}{\partial \alpha_k'} = 0; \quad \frac{\partial \Sigma w_j^2}{\partial \beta_k'} = 0 \quad 5.35$$

The above equations can be solved for α_k' and β_k' . Hence the estimates of α_k and β_k can be obtained. The fitting was achieved using a desk micro-computer (Apple IIe). The program flow sheet and its listing are given in Appendix A.

5.4.2 Fourier Transformation

On substituting for $\Delta\gamma(t)$ from Eq. 5.29, Eq. 5.22 becomes:

$$\epsilon = \frac{\text{F.T. } (\Sigma \alpha_k e^{-\beta_k t})}{\text{F.T. } (\Delta A(t)/A)} \quad 5.36$$

If $f(t)$ is a function of t , then the F.T. will be given by

$$\text{F.T.}[f(t)] = \int_{-\infty}^{\infty} f(t) \cdot e^{-i\omega t} dt \quad 5.37$$

thus the Fourier Transformation of $\Delta\gamma(t)$ is:

$$\int_{-\infty}^{\infty} \Delta\gamma(t) e^{-i\omega t} dt \quad 5.38$$

since

$$\Delta\gamma(t) = 0 \text{ for } t < 0$$

and

$$\Delta\gamma(t) = \sum \alpha_k e^{-\beta_k t} \text{ for } t \geq 0$$

Then,

$$\text{F.T.}[\Delta\gamma(t)] = \int_{-0}^{\infty} \sum \alpha_k e^{-\beta_k t} e^{-i\omega t} dt \quad 5.39$$

On solving, gives

$$\text{F.T.}[\Delta\gamma(t)] = \sum \frac{\alpha_k}{\beta_k + i\omega} \quad 5.40$$

and similarly

$$\text{F.T.}[\Delta A(t)/A] = \frac{F_\alpha}{i\omega}$$

where

$$F_\alpha = \Delta A/A \quad 5.41$$

Thus

$$\epsilon = \frac{\sum \frac{\alpha_k}{\beta_k + i\omega}}{\frac{F_\alpha}{i\omega}} = \frac{1}{F_\alpha} \sum_{k=1}^n \left(\frac{i\alpha_k \omega}{\beta_k + i\omega} \right) \quad 5.42$$

Multiplying the top and bottom by $(\beta_k - i\omega)$ and simplifying gives:

$$\epsilon = \frac{1}{F_\alpha} \sum \left(\frac{\alpha_k \omega^2}{\beta_k^2 + \omega^2} + i \frac{\alpha_k \beta_k \omega}{\beta_k^2 + \omega^2} \right) \quad 5.43$$

Therefore

$$\epsilon' = \frac{1}{F_\alpha} \sum_{k=1}^n \frac{\alpha_k \omega^2}{\beta_k^2 + \omega^2} \quad \text{and} \quad \epsilon'' = \frac{1}{F_\alpha} \sum_{k=1}^n \frac{\alpha_k \beta_k \omega}{\beta_k^2 + \omega^2} \quad 5.44$$

ϵ' and ϵ'' are calculated using a desk micro-computer. The listing of the program is given in Appendix B.

5.5 EXPERIMENTAL TECHNIQUES FOR DETERMINING SURFACE RHEOLOGY

Two different experimental techniques were used to determine the dilatational rheology:

- a. the expanding drop technique;
- b. the Wilhelmy plate technique.

5.5.1 The expanding drop technique

The apparatus for this technique is shown schematically in Fig. 5.10. Here a small drop was produced at the tip of a capillary tube by means of a calibrated syringe pump. The drop was connected to a sensitive pressure transducer, which was used to monitor the changes in surface tension values of the drop as it reached equilibrium. The cell containing the drop was small and its temperature and humidity were carefully controlled. Using the syringe pump a small amount of the liquid was added to the drop suddenly which caused a change in surface area, with a corresponding change in the surface tension of the drop. The pressure difference as given by the Laplace equation is

$$\Delta p = \frac{2\gamma}{r} \quad 5.45$$

where Δp = pressure , and

r = radius.

This shows that small changes in p will be most accurately determined when the drop is approximately hemispherical. The changes in internal pressure were recorded continuously until the drop reached its equilibrium state. The apparatus was interfaced to the computer.

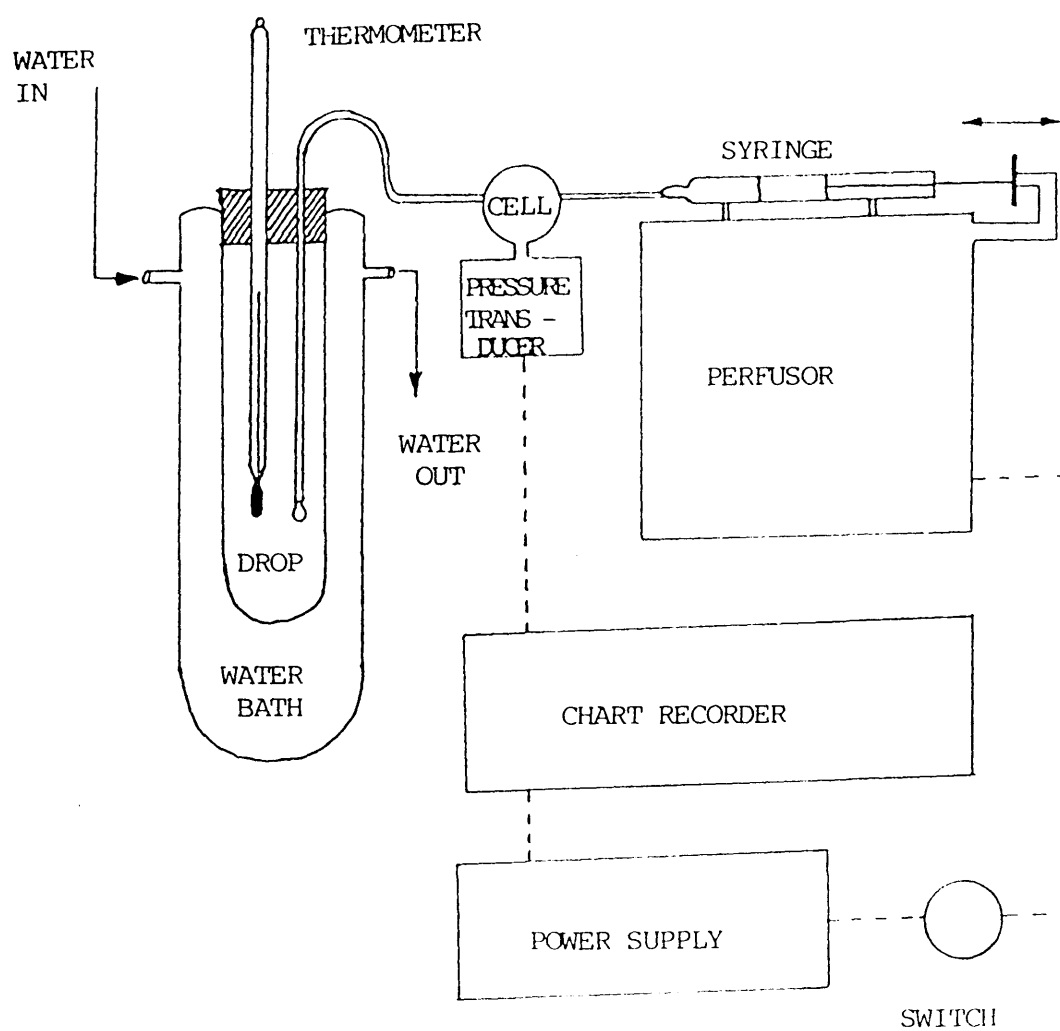


Fig. 5.10 Schematic presentation of the expanding drop apparatus

5.5.2 The Wilhelmy plate technique

The details of this apparatus are given in Chapter III. The protein solution was poured into the trough and left to equilibrate (~16 hrs). During the equilibration period the surface tension was measured continuously with time in order to make sure that equilibrium had been reached. The expansion of the surface was achieved by simultaneously moving both of the motor driven barriers. This ensured a uniform expansion of the surface. The Wilhelmy plate was placed in the centre of the trough and perpendicular to the barriers in order to minimise resistance to surface flow during expansion. The increase in surface tension and its decay with time were then continuously monitored and recorded on the chart recorder. A typical decay curve is shown in Fig. 5.11.

5.6 RESULTS

Dynamic surface tension measurements on adsorbed monolayers of Human Serum Albumin at air/liquid and oil/liquid interfaces were determined as a function of concentration and pH. The decay data were fitted to the analytical expression given above. For the system used here two exponentials were found to be sufficient to fit the data within experimental error. Fig. 5.11 shows the fitted line through the experimental points. It can be seen that a good fit is obtained, thus confirming the validity of the equation used and indicating the existence of at least two dominant processes. The values of the Fourier transformation of the analytical expression then yielded values of ϵ' and ϵ'' . The existence of two distinct relaxation processes is also shown by the Cole-Cole plots, Fig. 5.12. Here ϵ' is plotted

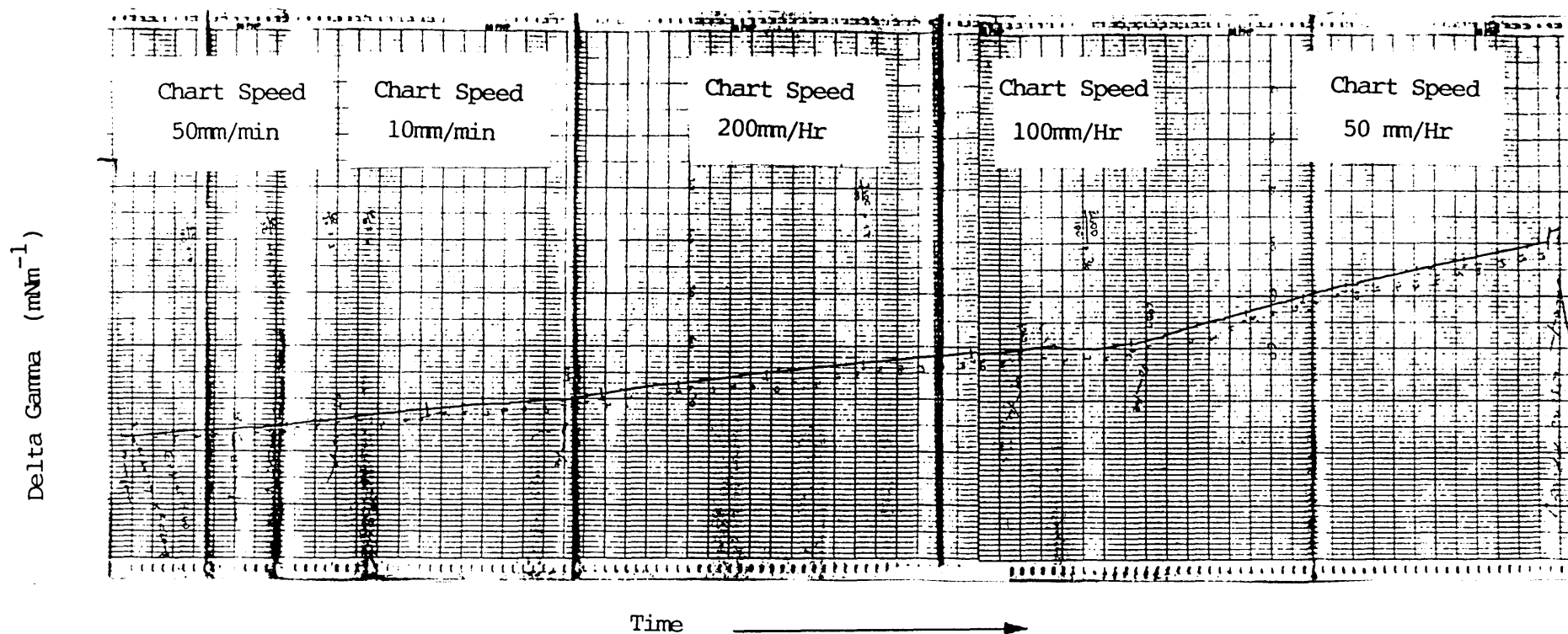


Fig. 5.11a Surface tension decay function ($\Delta\gamma$ -t) of human serum albumin. The concentration is 1×10^{-4} wt% at pH7.4. The kinks are due to change in chart speed.

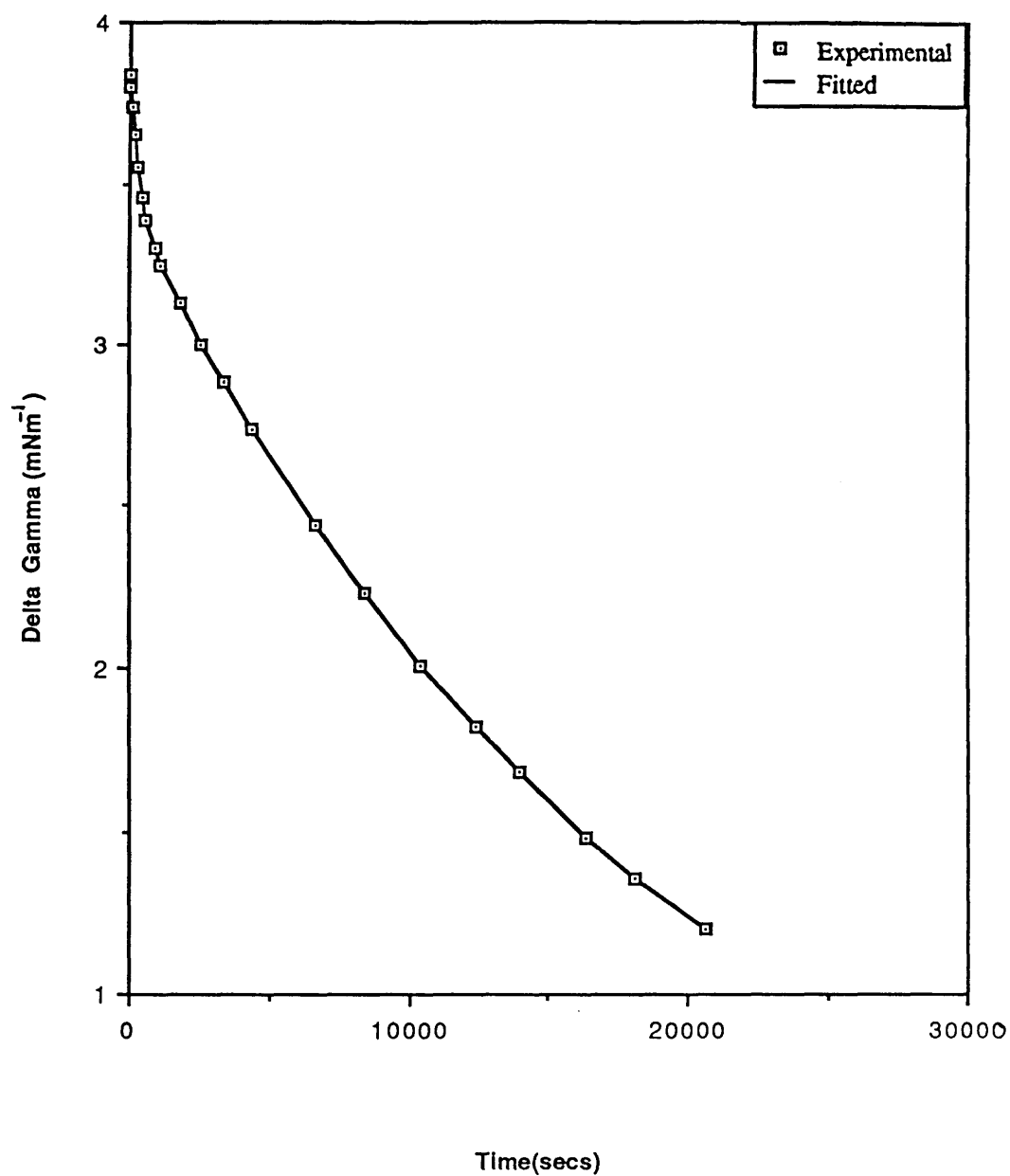


Fig. 5.11b Comparison of the dynamic surface tension data obtained by the stress relaxation experiments with the data obtained by the analytical expression for the system described in Fig. 5.11a.

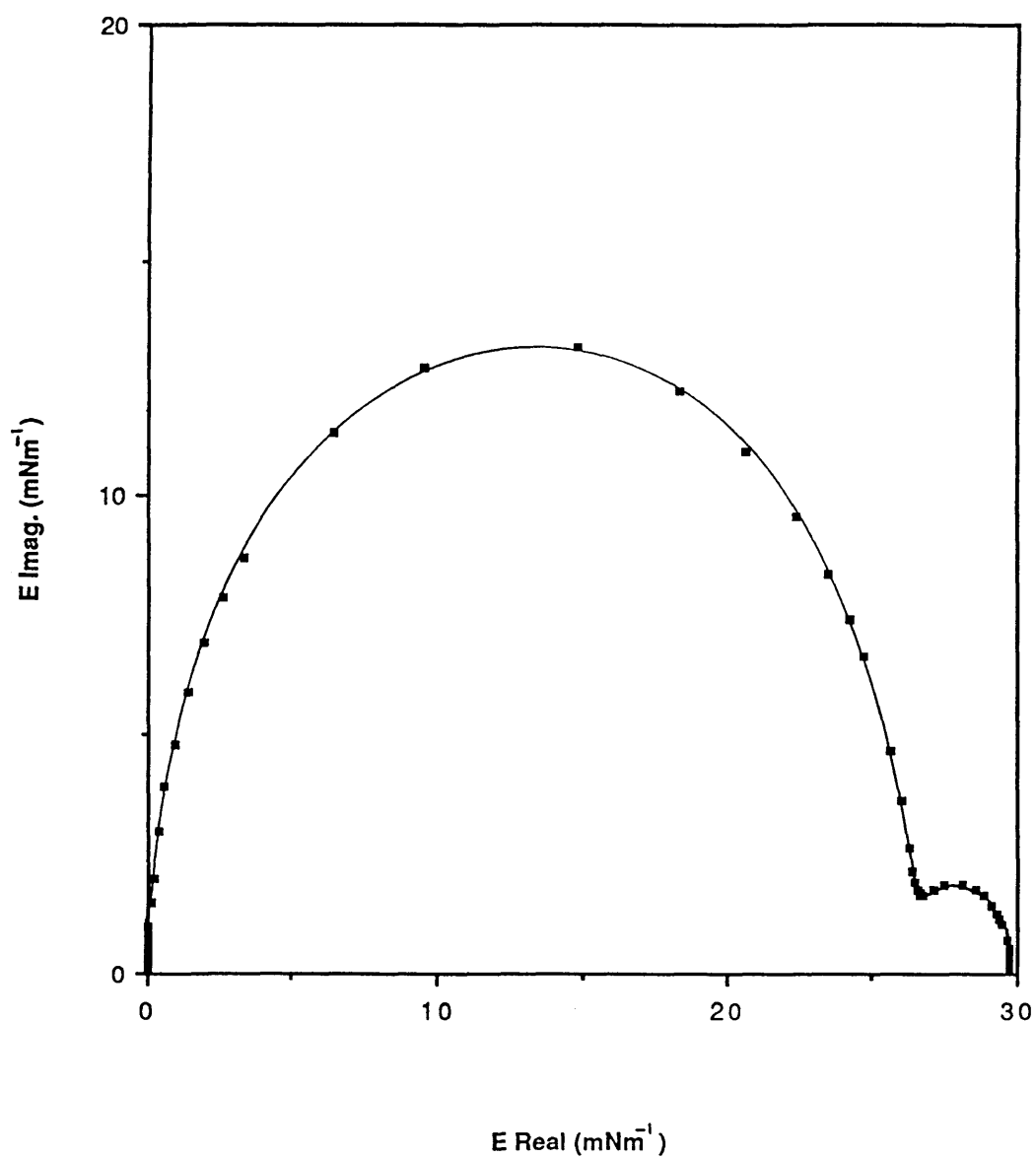


Fig. 5.12 Values of ϵ' plotted against ϵ'' as obtained by Fourier transformation of surface tension decay function for human serum albumin at pH7.4.

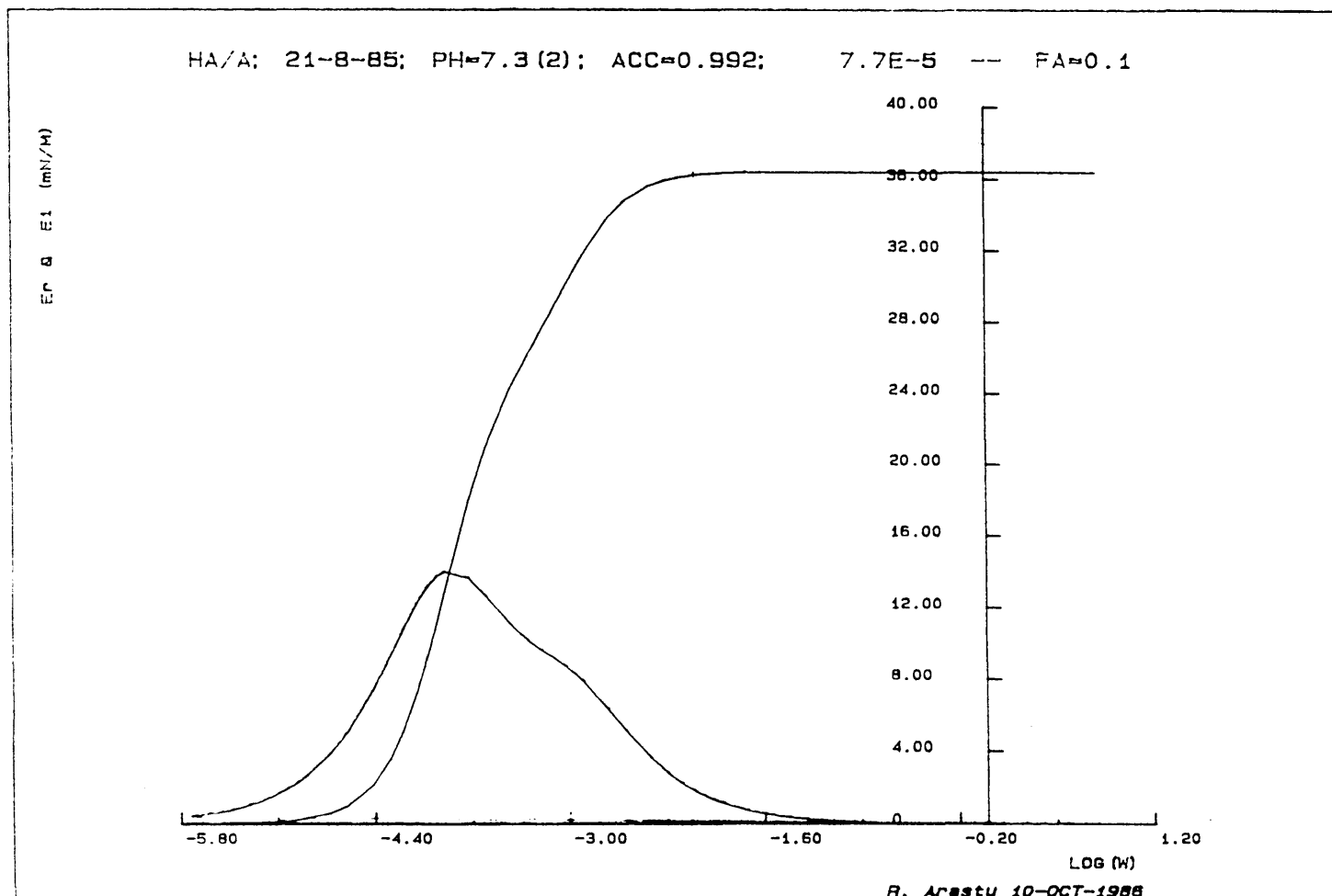


Fig. 5.13 Values of ϵ' and ϵ'' plotted against log frequency.
 $C_b = 7.7 \times 10^{-5}$ wt% when pH = 7.4 and $\Delta A/A = 0.1$ cm.

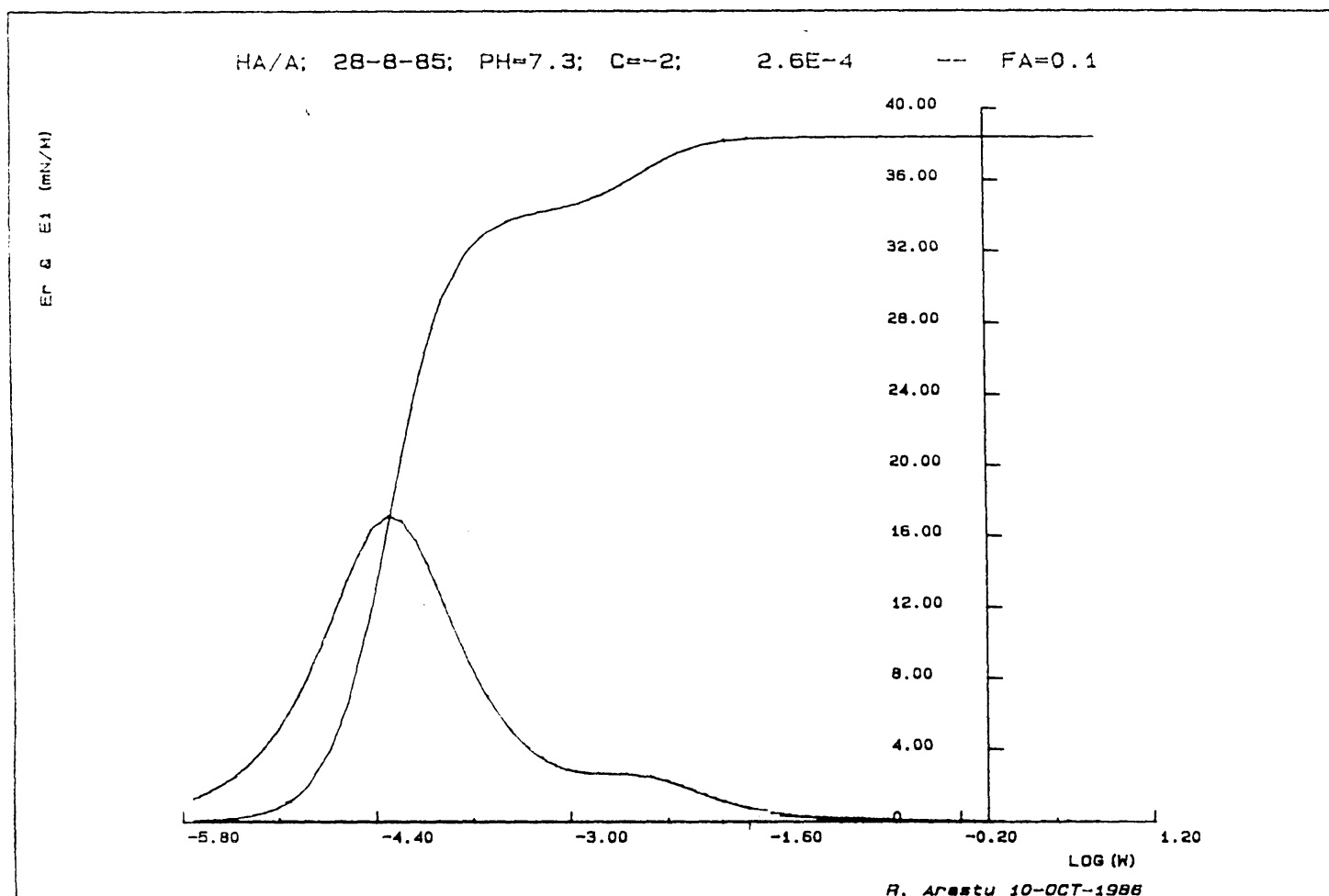


Fig. 5.14 Values of ϵ' and ϵ'' plotted against log frequency.
 $C_b = 2.6 \times 10^{-4}$ wt% when pH = 7.4 and $\Delta A/A = 0.1$ cm.

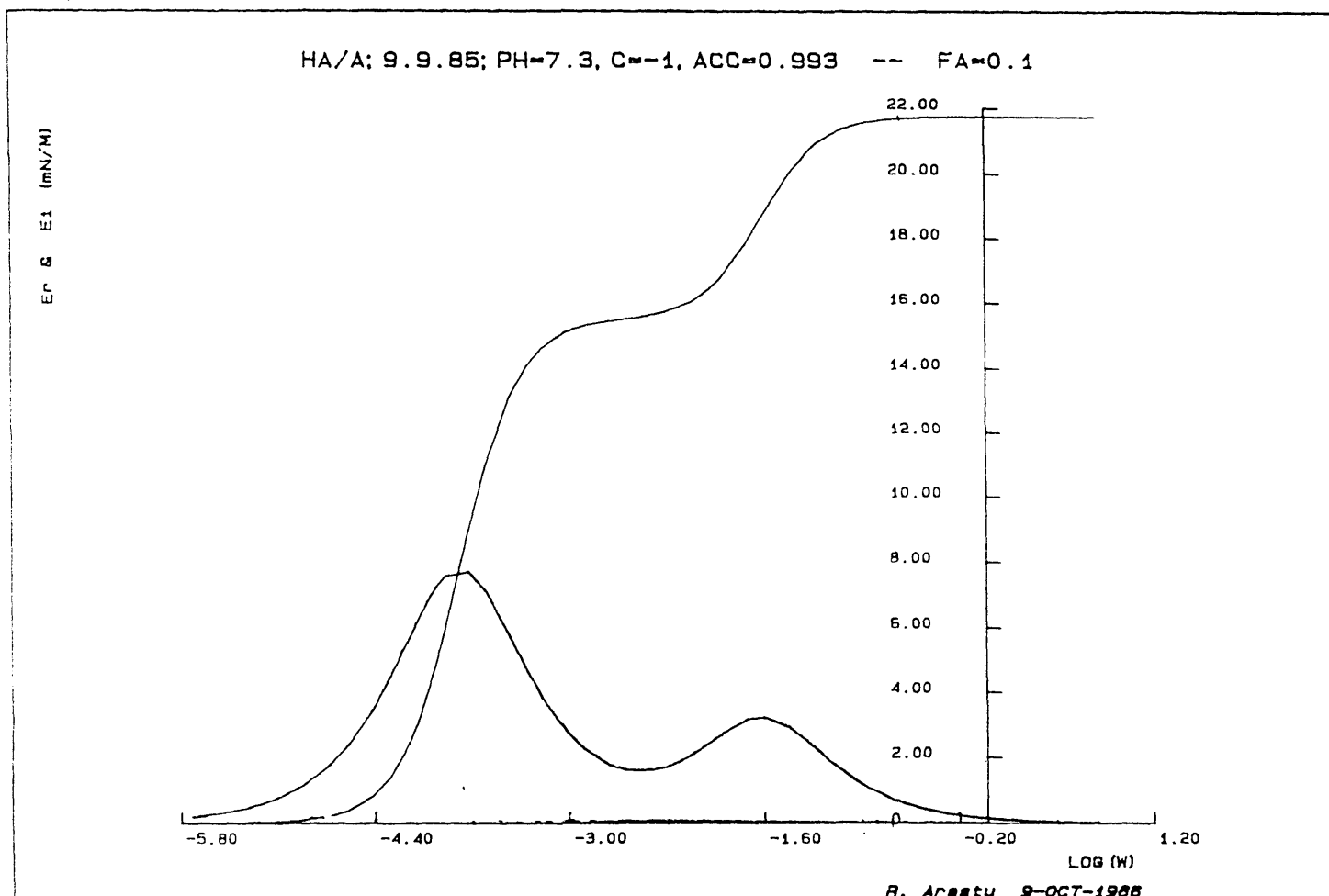


Fig. 5.15 Values of ϵ' and ϵ'' plotted against log frequency.
 $C_b = 4.2 \times 10^{-3}$ wt% when pH = 7.4 and $\Delta A/A = 0.1$ cm.

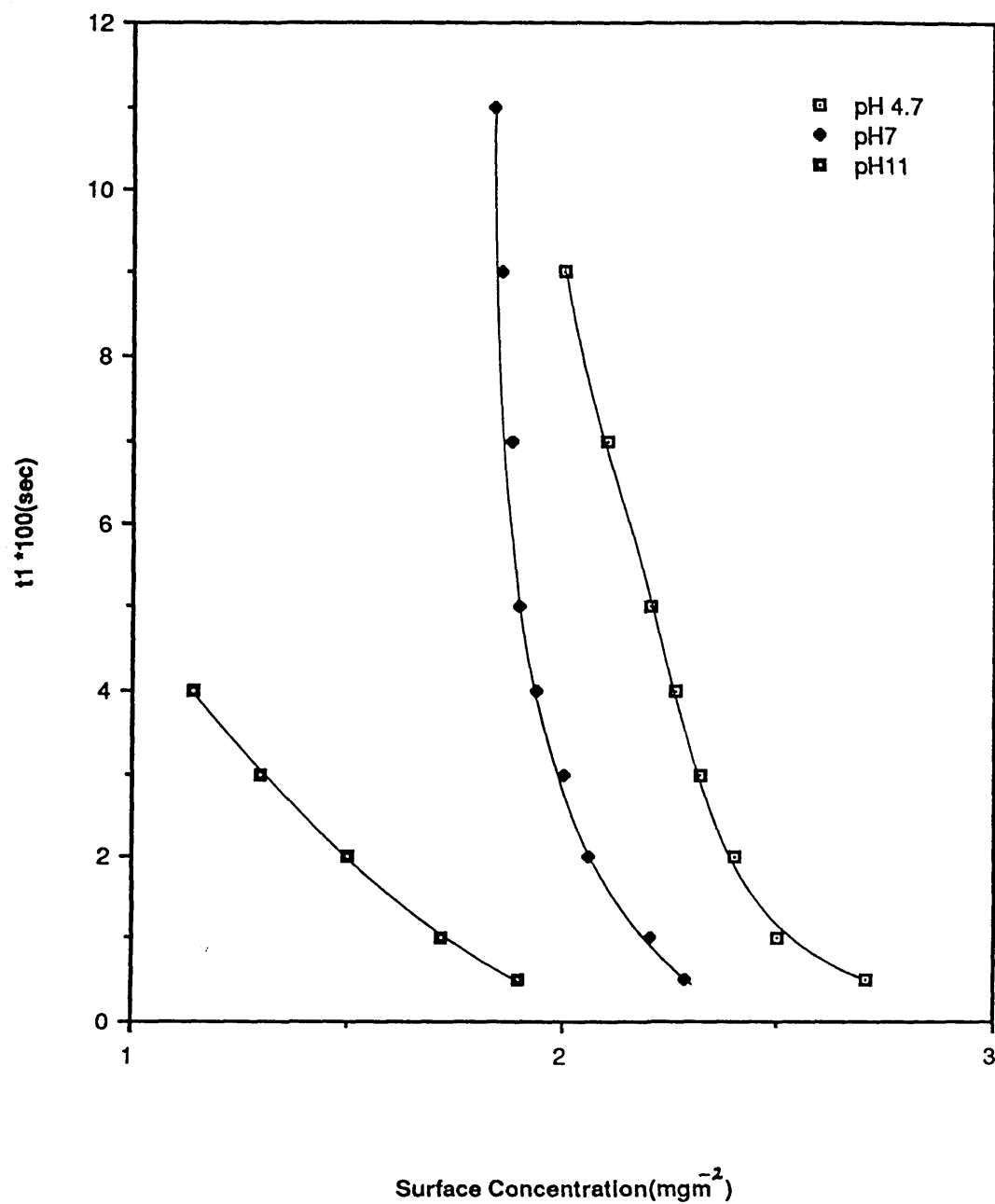


Fig. 5.16 The variation of relaxation time τ_1 as a function of surface concentration for different values of pH at air/water interface.

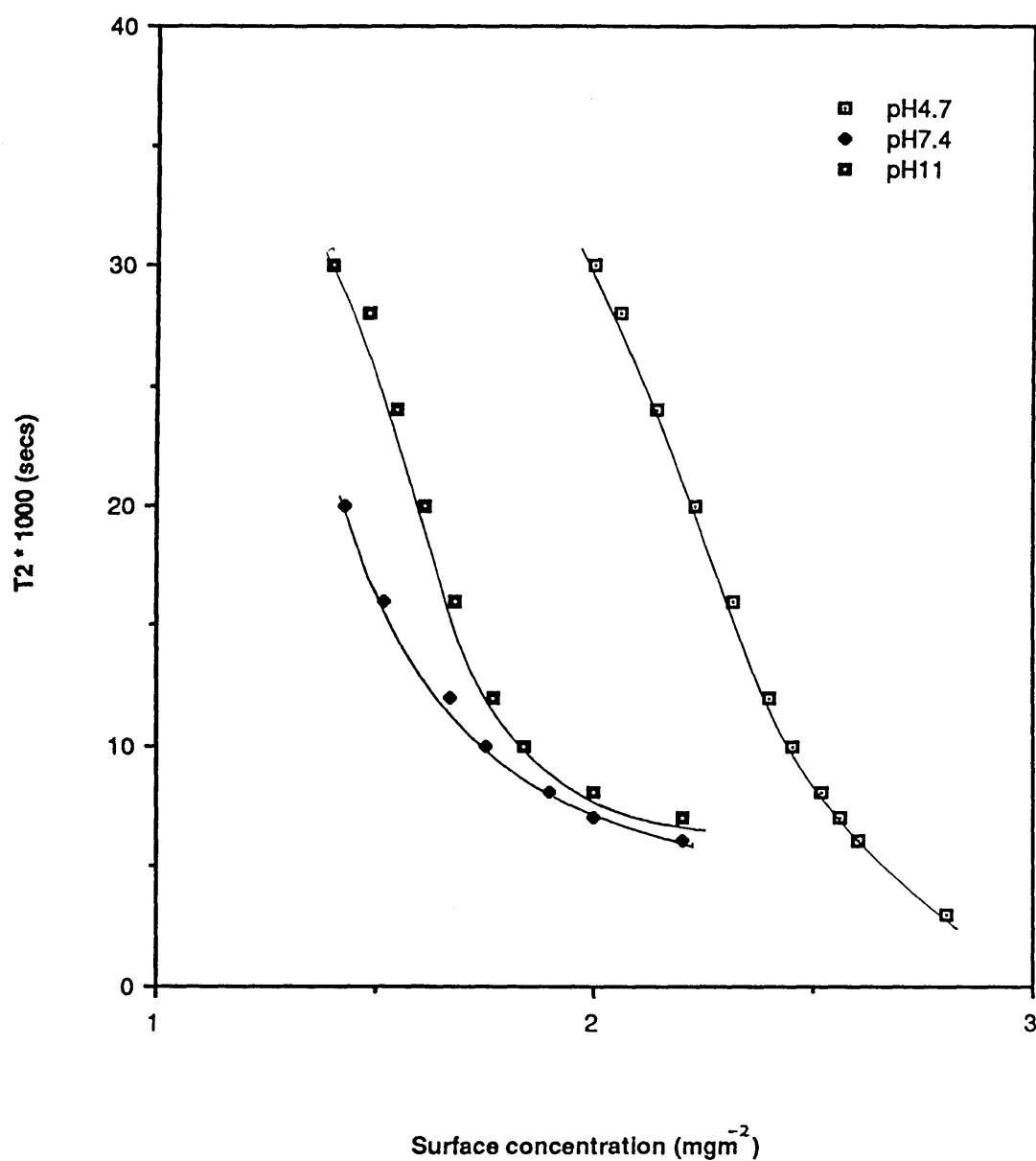


Fig. 5.17 Changes in the values of τ_2 as a function of surface concentration for different values of pH at the air/water interface.

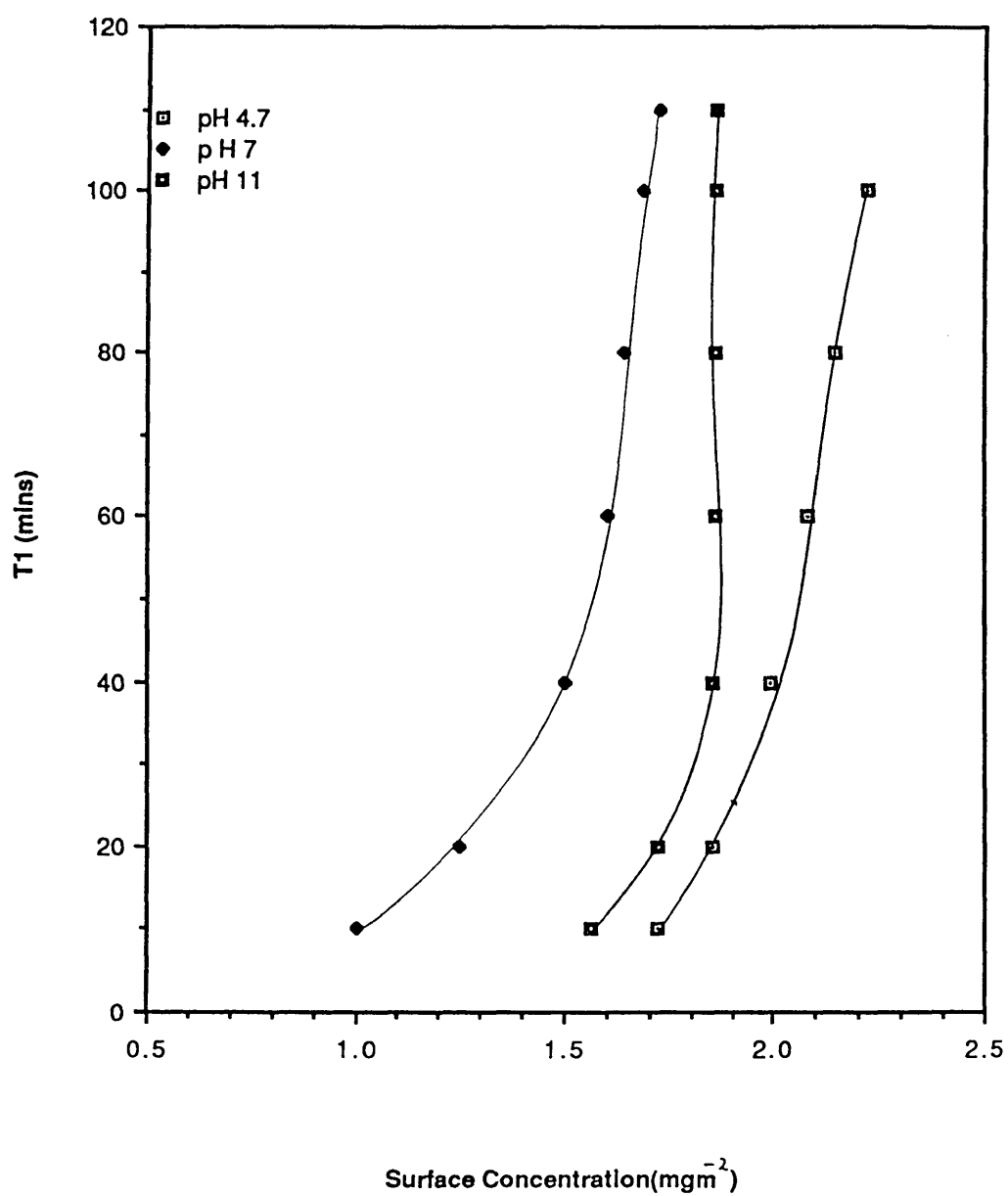


Fig. 5.18 The relative changes in the values of τ_1 with surface concentration are given for different values of pH at the oil/water interface.

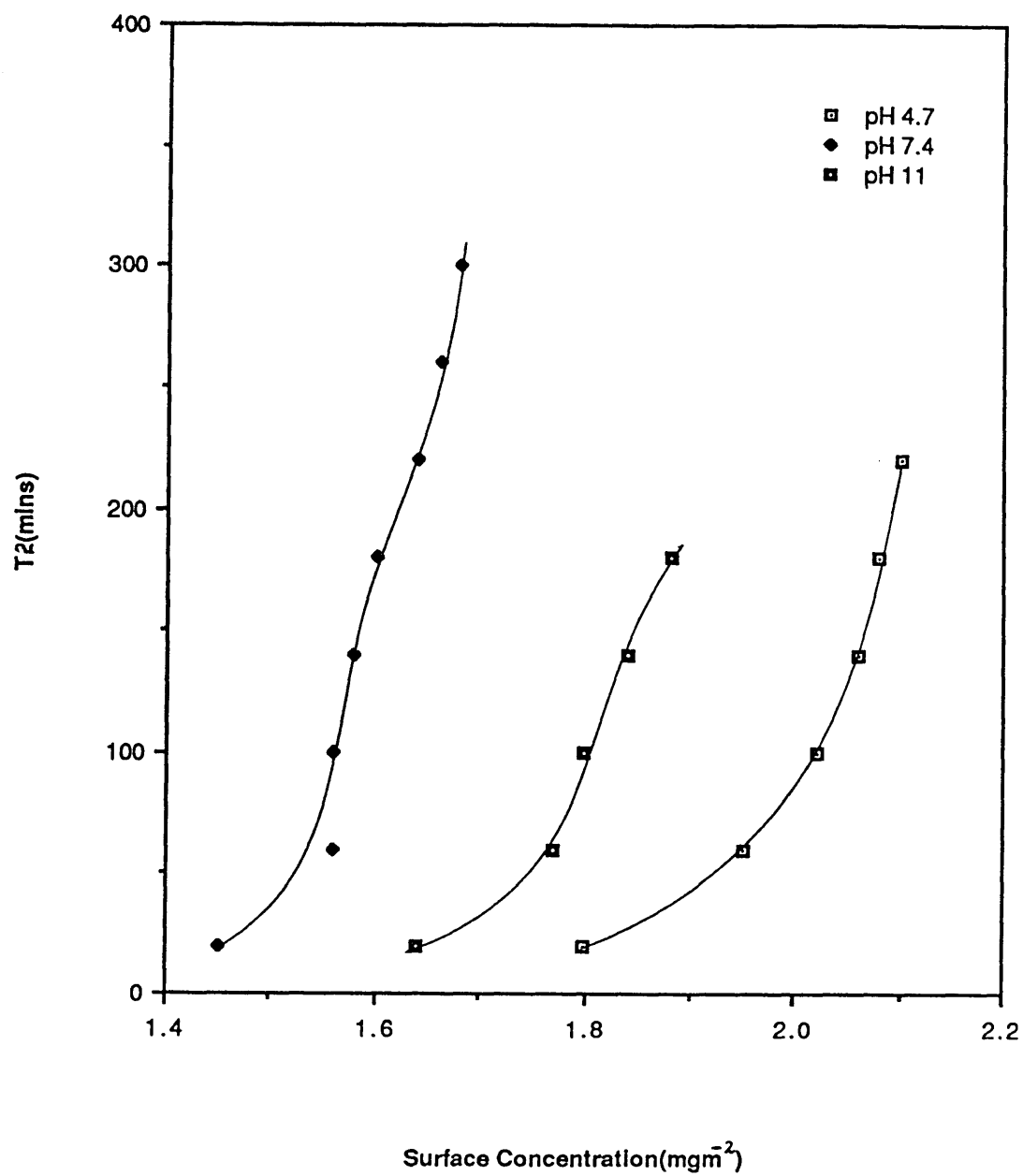


Fig. 5.19 The variations of the values of τ_2 with surface concentrations are given for different values of pH at the oil/water interface.

against ϵ'' . If a single process is responsible for the relaxation then a semicircle is expected. Hence the figures deviate from a semicircle indicating the existence of more than one process. The entire viscoelastic spectrum is shown in Figs. 5.13, 5.14 and 5.15 as a function of concentration. The characteristic times, τ_1 and τ_2 , of the relaxation processes thus obtained reflect the conformational changes that occur in the interfacial film.

The effect of surface concentration on τ_1 is shown for different values of pH in Fig. 5.16 for the air/liquid system. It is seen that with increase in bulk concentration the characteristic time decreases for all the pH values. The second process which has a longer characteristic time, τ_2 , is shown for different values of pH as a function of concentration at the air/liquid interface in Fig. 5.17. It is clear from the figure that τ_2 decreases with increase in concentration for the different pH values.

At the oil/liquid interface it is seen that the characteristic time τ_1 unlike the values at the air/liquid interface, increases with increase in surface concentration and is shown in Fig. 5.18 for different values of pH. The effect of concentration on τ_2 is shown in Fig. 5.19 for the same values of pH. It is seen that the values of τ_2 also increase as the concentration increases for all the pH values.

5.7 PHENOMENA OCCURRING DURING RELAXATION

The determination of the modulus gives information about the various processes which occur at an interface. In order to relate the relaxation parameters to actual physical or chemical phenomena so

that the molecular conformation of the molecule could be understood, it is essential to distinguish the various processes that occur during relaxation experiments. They are:

- a. Adsorption of molecules into the film from the bulk.
- b. Modification or uncoiling of the molecules once they have become part of the film. Molecules will adopt a configuration which reduces the surface free energy of the system.
- c. Aggregation or cross-linking of uncoiled molecules.
- d. Relaxation of stresses within the film by rearrangement of molecules in the surface into a less strained configuration.

Important information about adsorbed films can be gained by determining the initial rate of arrival of molecules at an interface as the adsorption here is irreversible and the activation energy is zero. Ward & Tordai (1946) derived an expression by which the initial surface concentration, Γ , can be related to the diffusion coefficient D and is given by the equation

$$\Gamma = 2C_b \left(\frac{Dt_o}{\pi} \right)^{\frac{1}{2}} \quad 5.46$$

where C_b = bulk concentration and t_o = time of adsorption. For that part of the adsorption curve, where the relationship Γ vs. $t_o^{\frac{1}{2}}$ is linear, the slope is equal to D . This holds only in the very early stages of adsorption where the substance once adsorbed, denatures and remains in the film.

Eq. 5.46 can only be used if Γ is known. Therefore for substances whose Γ is not known directly, the diffusion coefficient can be

determined by using the expression derived by Bendure (1971) for the reduced surface pressure $(\gamma_o - \gamma)/C_b$, viz:

$$\left(\frac{\gamma_o - \gamma}{C_b}\right) = 2RT \sqrt{\frac{\overline{Dt}}{\pi}} \quad 5.47$$

where C_b = bulk concentration;

R = gas constant;

T = absolute temperature;

γ_o = surface tension of pure system;

γ = surface tension of adsorbed film.

This equation predicts that a plot of the reduced surface pressure as a function of the square root of the adsorption time should be linear with a slope depending on the diffusion coefficient.

The bulk diffusion coefficient can also be estimated from the viscoelastic properties of the film. According to Luccassen & Giles (1975), for a diffusion controlled process, the diffusion coefficient is related to the characteristic frequency of the relaxation process by

$$\omega_o = \frac{D}{2} \left(\frac{dC_b}{d\Gamma}\right)^2 \quad 5.48$$

where ω_o = characteristic frequency in rad/sec.;

D = diffusion coefficient;

C_b = bulk concentration;

Γ = surface concentration.

If the faster of the two processes observed here is diffusion controlled then the above equation should determine the diffusion coefficient once surface concentration is known. Thus for a bulk

concentration of 7.7×10^{-5} wt% the value of the diffusion coefficient obtained is $1.2 \times 10^{-15} \text{ cm}^2 \text{ sec}^{-1}$ which is much smaller than the literature values.

The values of D for HSA determined by quasielastic light scattering spectroscopy have been given in the literature as $6 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ (Phillies *et al.* 1971). The characteristic diffusion time τ' may be determined by using the Einstein relation which is

$$\tau' = \delta^2 / 2D \quad 5.49$$

where δ is the largest dimension of the molecule ($140 \text{ \AA}$).

From this relation τ' was calculated to be $1.6 \text{ } \mu\text{s}$ (Schaaf *et al.* 1987).

We may compare τ' with the experimentally determined values of τ_1 and τ_2 . Even the short relaxation time τ_1 is a factor of 10^6 longer than τ' . This suggests that τ_1 is associated with conformational changes in the molecules which take place after the molecules have diffused to the surface. The relaxation due to diffusion is too fast to be detected by the current equipment.

5.8 NATURE OF THE CONFORMATIONAL CHANGES IN THE HSA MOLECULE

In order to understand these effects it is necessary to consider the nature of the unfolding of the HSA molecule in solution and on the surface. The structure of the molecule was given in Fig. 2.4 from which it is seen that the presence of the disulphide bridges means that only in the presence of denaturing agents is it possible for the molecule to assume a single threadlike structure. The loops indica-

ted by 1 to 9 in the figure exist at neutral pH and as a result of the overall charges on the loops they associate to form globular regions or domains. A suggested structure for albumin at pH7 is shown in Figs. 5.20a and 5.20b. The domains are folded together in the native state. On adsorption, initial unfolding will consist of an opening of the structure to reveal the domains. This opening up will depend on the concentration with many molecules remaining in their native state at high concentrations.

The pH of the medium affects the conformation in the bulk solution. At pH4.7 where the molecule has equal numbers of positive and negative charges. It is most compact and most insoluble. Adsorption is therefore high. At high values of the pH the molecule is in a progressively more expanded state. This has been demonstrated by the activity of the tyrosine residues. Eleven of the total of 18 or 19 are exposed at pH11.4. The S-S bonds are extremely stable between pH4.7 and pH7 and do not react even in strong reducing agents. As the pH increases they gradually become more reactive but full reduction even at high pH is only possible in the presence of denaturing agents such as 8M Urea (Kauzman & Simpson, 1953).

Under acid conditions expansion of the molecule first into domains and later into loops occurs. At pH2 all carboxyl groups will be protonated and a high charge density of 1-2 positive charges per 10 amino acid groups occurs on each of the 9 loops.

Clearly the conformation in the bulk will affect the behaviour of the molecules after adsorption. The structure of the adsorbed film will thus vary with pH and the viscoelastic properties will change as a consequence.

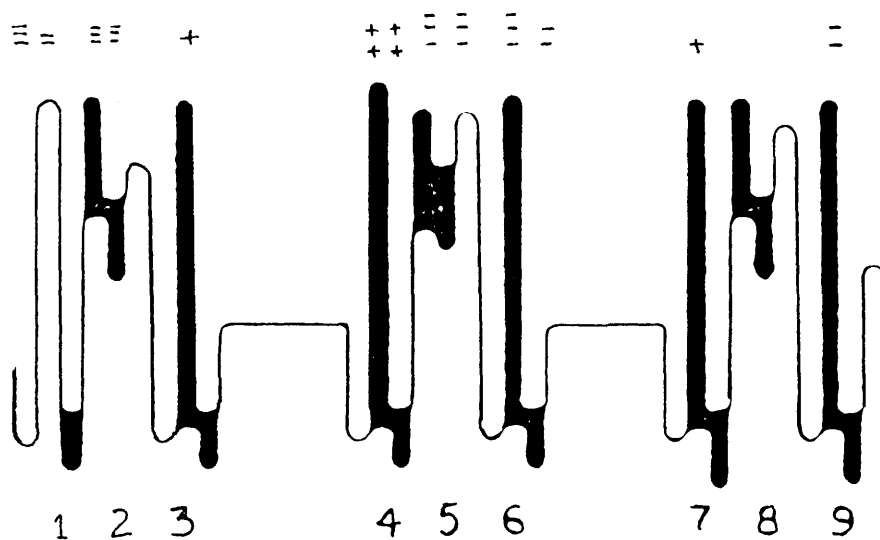


Fig. 20a On the basis of Figs. 2.3 and 2.4 distribution of net charges in the double loops of albumin at pH 7.0 assuming no carboxyl or imidazole groups are protonated.

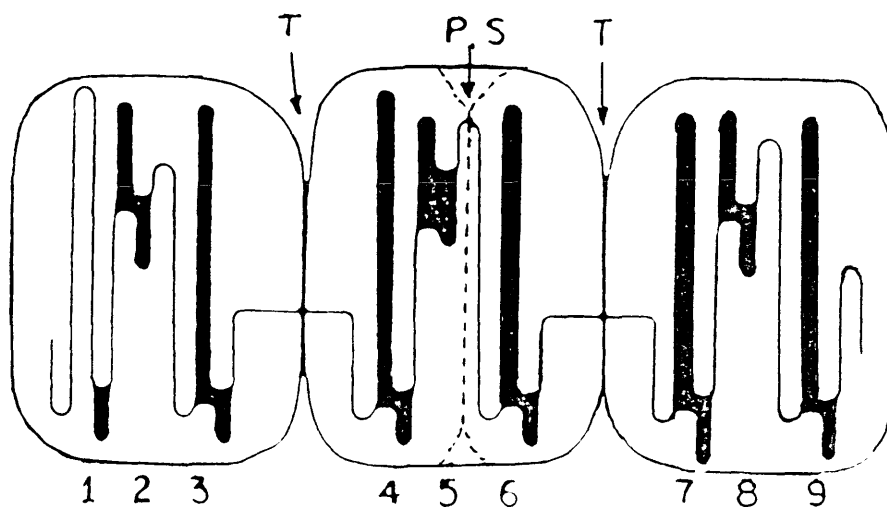


Fig. 20b A suggested grouping of loops of albumin into globular parts or domains at pH 7.4. T = trypsin; P = pepsin; S = subtilisin. The evidence for the division between loops 5 and 6 shown as a broken line is less since then it is for the other two separations.

5.8.1. Films formed at the air/water interface

From Fig. 5.16 the characteristic time, τ_1 for the faster process is seen to be less at pH11 than at pH7.4 and pH4.7. These results are consistent with our knowledge of conformations in the bulk. At pH11 the molecules adsorb in a state in which the domains and to some extent the loops are already open and little time is needed to complete the process. At pH4.7, however, the molecule starts to unfold by separation of the domains, a process which will continue as long as space is available. The behaviour is similar at pH7.4 but as the molecule is slightly more accessible to solvent at this pH, it is less tightly held together and the relaxation occurs more readily. A qualitative model of the adsorbed molecules is shown in Fig. 20 for different values of the pH.

The relaxation time decreases with increase in surface concentration in all cases as a result of the decrease in the space available for expansion at high concentration. The surface pressure of the molecules already present on the surface hinders unfolding and many molecules adsorb in their native form. Evidence for this is obtained by the desorption of some molecules from the surface film when it is compressed, see Chapter Three.

The second relaxation process has a very long characteristic time, τ_2 . Aggregation phenomena give short times as shown by Veer *et al.* (1973) therefore τ_2 cannot be due to this. In any case although many proteins such as muscle proteins and the structural proteins of membranes, show strong aggregation properties, HSA is unlikely to form aggregates with itself although it frequently associates with other molecules. The most likely process is thus the slow rearrange

ment of the molecules allowing them to take up less strained configurations. This will involve the movement of whole domains or even native molecules in the surface at the isoelectric point and τ_2 has its highest value. Since the charges on HSA render it most soluble at pH7.4, the relaxation could be assisted by parts of the molecule being drawn back into the water phase. At pH11 there are likely to be many long hydrophobic chains adsorbed at the surface as well as domains. Entanglement of the molecules could also occur readily. The release of strain is less assisted by the bulk phase as a result. Hence τ_2 lies between the values at pH4.7 and pH7.4.

5.8.2 Films formed at the oil/water interface

Relaxation at the oil/water interface takes place much faster than at the air/water interface. τ_1 is a factor of 10 faster and τ_2 a factor of 2. In addition τ :A curves show that the film is more expanded. Both of these effects arise as a result of the additional interactions with the oil phase which will depend on the degree of hydrophobicity of the molecule. HSA is most hydrophilic at the physiological pH7.4. Hence its interaction with the oil phase will be least at this pH and the relaxation time correspondingly longer. This is because it takes time for the hydrophobic subunits of the chains to become exposed to the oil phase. At pH4.7 as the molecule is most compact the degree of unfolding is least and hence it requires the least time. This results in a higher surface concentration. At pH11, some of the disulphide bridges on some molecules are attacked in the bulk and the molecule is considerably expanded. On adsorption, hydrophobic moieties will be drawn into the oil phase and the molec

ules are able to contract somewhat when compared with their behaviour at the air/water surface. This is confirmed by the 5-10% increase in Γ at the oil/water interface. A suggested model of the adsorbed molecules is shown in Fig. 5.21.

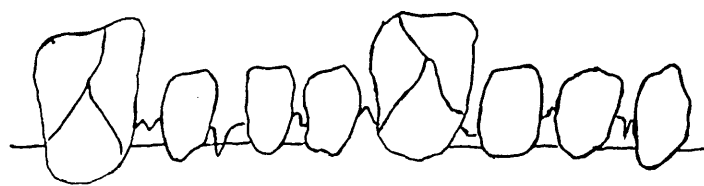
As the surface concentration increases the values of τ_1 rise. This is in contrast to the behaviour at the air/water interface. It is a consequence of the fact that almost all of the molecules arriving at the interface are able to unfold to some extent because of the interaction with the oil.

τ_2 , the slow process, is again associated with relaxation of stresses in the film. The process is now assisted by the presence of the oil and hence the characteristic times are lower. The film is likely to have a more compact structure at the isoelectric point even though most molecules will be unfolded to some extent. τ_2 will be least since little readjustment needs to take place. At pH11 the molecule is most expanded and parts will be drawn into the oil phase providing an increased number of possible configurations to relieve the stresses. For pH7.4 the molecules are likely to retain some remnants of the globular structure and the rearrangement of these larger masses will require a longer time. All relaxation times are found to increase with concentration since interactions between the molecules causes the adjustment to slow down.

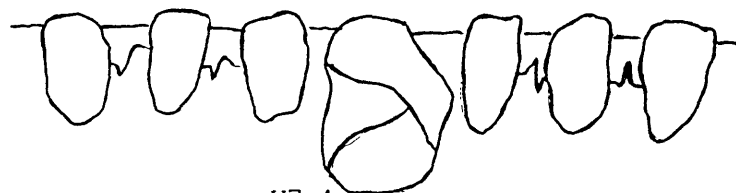
5.8.3 Measurement of η_d from stress relaxation experiments

From the imaginary part of the modulus ϵ , values of η_d may be determined for a particular frequency. It is given by

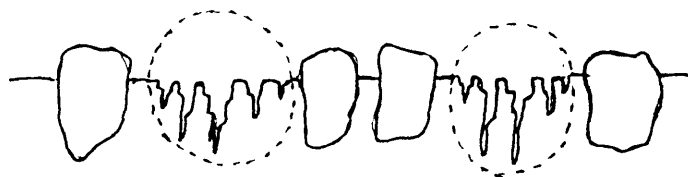
$$\epsilon'' = \eta_d \omega$$



pH4.7

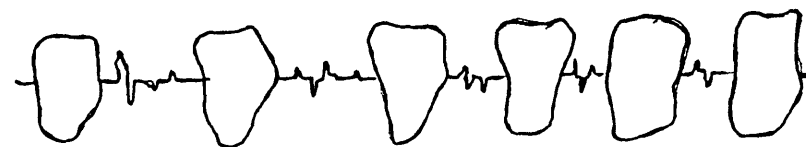


pH7.4



pH11

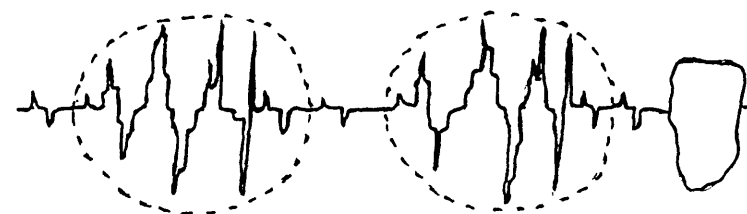
(a)



pH4.7



pH7.4



pH11

(b)

Fig. 5.21 Model of adsorbed molecules for different values of pH is shown for the (a) air/water interface and (b) oil/water interfaces.

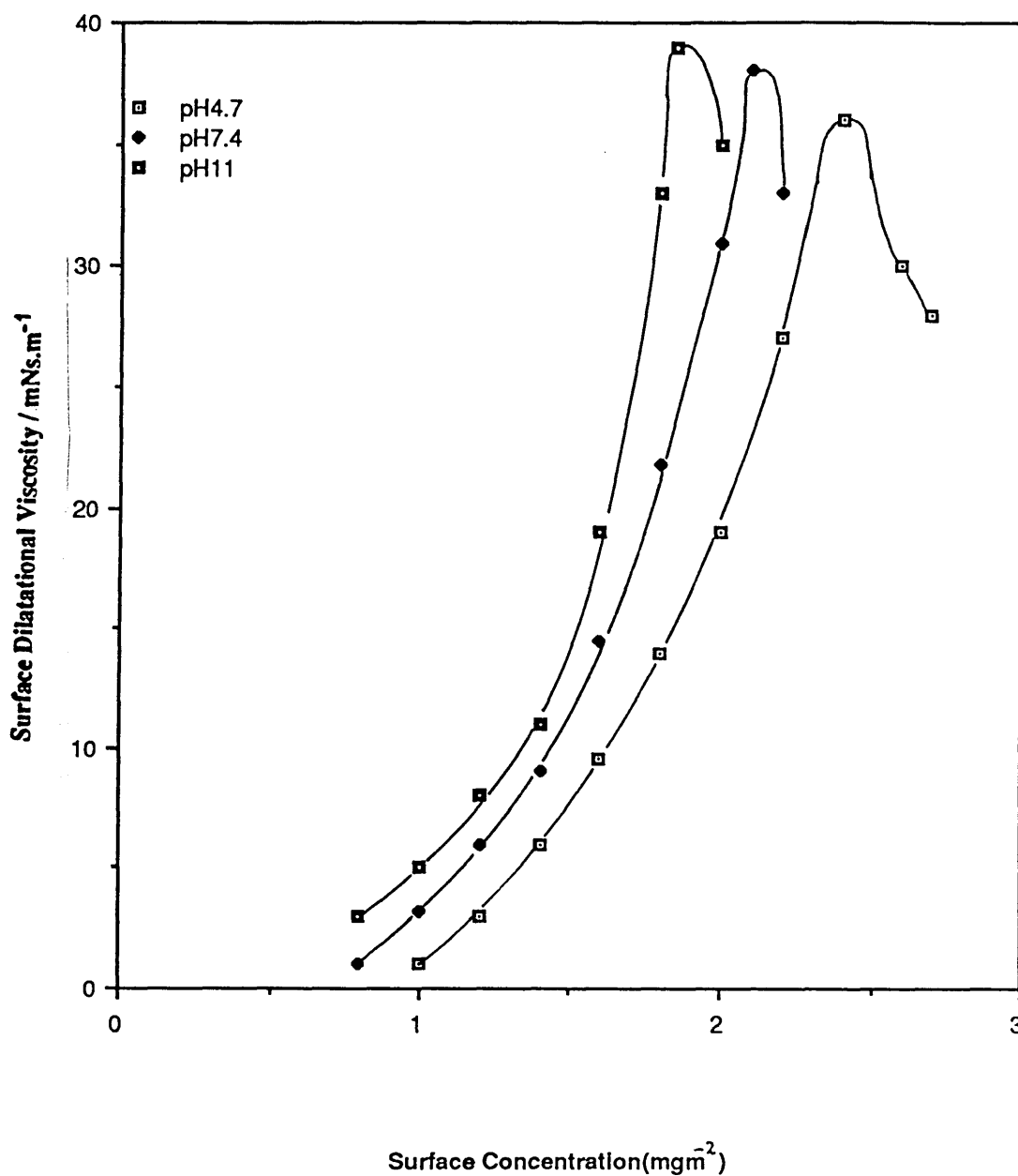


Fig. 5.22 The variations of surface dilatational viscosity (η_d) as a function of surface concentration for different values of pH is shown for the air/water interface. (Maximum is attained earlier for molecules in the unfolded state.)

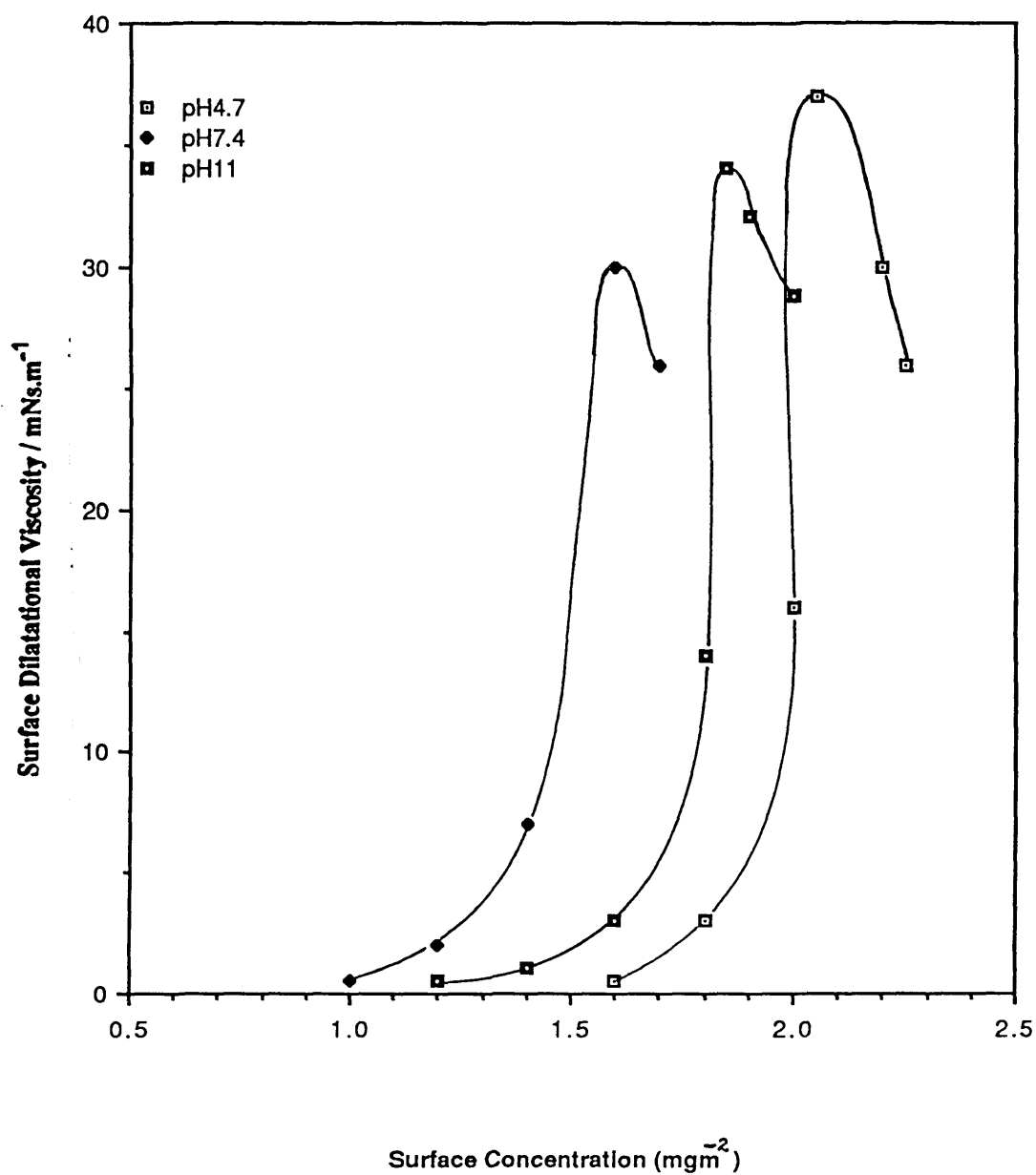


Fig. 5.23 The relative changes of surface dilatational viscosity (η_d) with surface concentration are given for different values of pH at the oil/water interface.
(Maximum is attained in the order of hydrophobicity.)

where ϵ'' is the imaginary part of the complex modulus. η_d decreases with frequency. It is to be expected that η_d should depend on the surface concentration and also on the state of the molecules in the surface film. The values calculated from the results obtained at the air/water interface for different values of the pH are given in Fig. 5.22. The curves increase with Γ and show a maximum value in each case. The results are consistent with the model of the interface suggested above. The more expanded the molecules the greater the number of segments in the interfacial region. Consequently the viscosity reaches its maximum at lower values of Γ .

At the oil/water interface loop formation into the oil phase influences the values of η_d , as shown in Fig. 5.23. At pH4 since the molecules are less unfolded than at other values of pH, η_d reaches its maximum at higher values of Γ . At pH11 the molecule is more compressible than at pH7, where some remnants of the globular structure remain. The values of η_d are similar at both interfaces.

CHAPTER SIX

CONCLUSION

6.1 ADSORPTION AT AIR/LIQUID AND LIQUID/LIQUID INTERFACES

From the study of the adsorption characteristics of a protein it has become clear that the time required for the adsorbed protein molecules to reach a steady state is much longer than that for low molecular weight compounds. The surface tension decreases over a long period after adsorption is complete indicating that slow adjustments in the structure of the monolayer must be taking place. The accompanying conformational changes in the adsorbed molecule have been systematically studied here by investigating the following surface properties.

1. Changes in the surface or interfacial tensions.
2. The interfacial concentration corresponding to a given bulk concentration.
3. The surface dilatational rheology.

All the above properties were measured as a function of the pH and bulk concentration at both the air/liquid and liquid/liquid interfaces.

6.1.1 Changes in the surface and interfacial tensions

The change in interfacial tension is a measure of the surface activity. From the changes in the surface tension values of an adsorbed film of human serum albumin it is seen that equilibrium is not attained until about 16 hrs after adsorption begins. This time dependent change is attributed to diffusion of molecules to the

surface and subsequent changes that occur in the conformation of the molecule. At a particular bulk concentration as soon as molecules arrive at the interface a certain degree of unfolding occurs which depends on the internal structure of the particular protein, and the molecules spread out to occupy a greater area. They orient so that the hydrophobic groups project into the apolar phase and the hydrophilic groups towards the polar phase. The initial rapid changes in the surface tension values are diffusion controlled.

As more molecules arrive at the interface and the surface concentration increases it becomes difficult for further adsorption and unfolding to occur. Changes in the interfacial tension values continue to occur but at a much slower rate until a compact monolayer is formed. Thermal motion in the compact layer causes further very slow adjustments to the structure.

As the bulk concentration of the protein solution is increased the equilibrium surface tension values of the adsorbed monolayers decrease at both the air/liquid and liquid/liquid interfaces until at a particular concentration the monolayer becomes saturated. Any further increase in the bulk concentration does not significantly affect the surface tension values.

As it is the surface concentration and area of the molecule at the interface which affects the surface and interfacial tension values, therefore any parameters which affect the configuration of the molecules should also cause measurable changes in the way in which the surface and interfacial tension varies. Thus, as was seen in Figs. 3.9 and 3.10 of Chapter Three, the pH of the solution affects the charge distribution on the molecule and hence the accessibility of sol

vent to the internal structure causing various degrees of conformational change with a resultant change in γ .

Proteins at the interface exist as fully or partially unfolded polypeptide chains in the α -helix configuration. The number of non-polar side groups extending into the hydrophobic phase and of polar side chains in the aqueous phase must be consistent with the steric configuration of the molecule. Lateral forces between side chains and the interactions of the side chains with the media also influence this conformation. The number of the hydrophobic and hydrophilic groups exposed depends on the pH of the aqueous phase. The effect is maximized for values of pH away from the isoelectric point where due to the absence of a net charge, the molecule exists in its most compact form.

In solution, the properties of the molecule depends on the degree of ionization. Since α -amino and carboxylic groups are used for the formation of the peptide bonds, therefore the polar groups of the amino acid side chains must provide the major contribution to the behaviour of the molecule in electrolyte solution. The groups in the side chains of the amino acid residues that contribute are the carboxyl group of glutamic and aspartic acids, the imidazole group of histidine, amino groups of lysine, phenolic hydroxyl groups of tyrosine, thiols of cysteine and guanidine residues of arginine. They will, therefore, carry a net positive or negative charge depending on their state of ionization.

As the charge on the protein molecule is a function of pH therefore variations in the value of pH will influence the conformation of the molecule. Hence the properties of the adsorbed film at both the

air/liquid and liquid/liquid interfaces will depend on the pH of the aqueous medium.

The unfolding of macromolecules is largely dependent upon the interfacial energy. For some liquid/liquid systems where the interfacial energy is low, the unfolding of the adsorbed molecules of protein is inhibited. As a result the adsorbed films in these systems are more compact than those formed at the air/liquid interface.

However, when organic liquid penetrates between the hydrocarbon chains in the hydrophobic residues of the molecules then the cohesion between the non-polar parts of the film forming molecule reduces. This causes the macromolecules to unfold and adopt a loose configuration at the surface. Such molecules tend to form expanded films far more rapidly at the liquid/liquid interface than at the air/liquid interface.

In solution when the degree of ionization is high, there is electrical repulsion between the charged groups of individual molecules. This causes the molecule to expand, thus changing the area occupied by the molecule at the surface. Repulsion between the molecule results in expansion of the whole film and assists the unfolding. Also, due to the solvation of carboxyl or amino groups the affinity of the molecule for the solvent is increased. This increases the tendency of the proteins to remain in solution, reducing the surface concentration. However, at the isoelectric point where the net charge is zero the molecule adopts a compact configuration. Therefore adsorption of molecules per unit area reaches a maximum. In addition, the affinity of the protein molecule for the solvent decreases and the interfacial film thus formed will be less hydrated and more concentrated.

At the liquid/liquid interface maximum adsorption is also observed at the isoelectric point. The adsorption, however, does reduce on either side of the isoelectric point but unlike the air/ liquid interface it is seen that the adsorption at pH 11 is higher than that at pH7.4. As it is the number of segments adsorbed per unit area that primarily determines the interfacial tension, those proteins whose molecular conformation permits maximum utilization of the available hydrophobic groups to penetrate the interface will decrease the free interfacial energy to the greatest extent. This is illustrated by comparing the interfacial tensions of solutins at pH 11 and pH7.4 at the liquid/liquid interface. At pH11 the molecule is more unfolded in solution than at pH7.4 and hence a large number of segments per unit area are adsorbed initially, resulting in a lower initial interfacial tension.

A marked difference in the adsorption characteristics is also observed with a change in non-polar media.

At a given pH, say pH11, the difference in adsorption between the air/liquid and liquid/liquid interfaces, as shown in Figs. 3.11 and 3.12, can be attributed to the difference in the interaction of the hydrophobic side chains with air and with the non-polar medium. The increased interaction with the non-polar phase causes further unfolding and expansion of the molecules in the adsorbed monolayer.

6.1.2 Interfacial concentration corresponding to a given bulk concentration

From the direct surface concentration measurements obtained by the radio tracer technique it is clear that the initial rate of arrival of the molecules at the interface is high and it gradually reaches

a plateau (see Fig. 4.9). The time required for the molecules to penetrate the interface, as calculated from the diffusion coefficient values, is found to be of the order of 10^{-6} sec. This shows that the initial rate of adsorption is very rapid and is diffusion controlled. The activation energy to adsorption is zero initially. Hence during the early stages of adsorption every molecule that collides with the interface is adsorbed and this adsorption is irreversible. However as the surface concentration becomes effectively constant the continued decrease in the interfacial tension values shows that the molecules in the monolayer are rearranging to form a condensed film. From the measurements of surface concentration which are obtained as a function of bulk concentration and pH, it is observed that after the initial rapid adsorption the rate of increase in the surface concentration is closely related to the dimension of the molecule in solution.

From the rate of adsorption obtained by the radiotracer results Figs. 4.10 and 4.11, it is observed that the maximum amount adsorbed is highest at the isoelectric point for both the air/liquid and liquid/liquid interfaces. This is because in the initial rapid adsorption the compact form of the molecules allows for greater numbers to arrive before the unfolding begins. At other values of the pH the amount adsorbed depends on the conformation in solution and the interaction with the apolar phase. This accounts for the differences which are observed in the behaviour at pH 7 and pH 11.

6.1.3 Surface dilatational rheology

The ultimate configuration adopted by the molecule on adsorption at an interface depends on the nature of the molecule itself. In the

case of β -casein, which approximates a random coil, the adsorption at the air/liquid interface for low surface coverages occurs with the peptide backbone lying in an all train configuration. As surface concentration increases, the adsorbed layer becomes close packed and loop formation becomes energetically favourable.

It is seen that the globular proteins are more efficiently packed than other proteins. This is due to the presence of disulphide bridges between the two cystein residues. These disulphide linkages impose considerable restraint on the structure of the molecule and the interaction between protein molecules in the adsorbed film, thus influencing the degree of molecular unfolding which is possible at the interface. In other words, the degree of unfolding affects the surface activity of the protein.

The relaxation time related to unfolding of the molecules is orders of magnitude greater than that for adsorption. The surface concentration is largely determined before unfolding begins. The degree of molecular unfolding at the interface is significantly influenced by the surface concentration of the adsorbed layer. The hydrophilic and the hydrophobic residues are preferentially located into the water and air phases respectively. Hence β -casein although able to unfold completely does not do so at high surface concentration at the air/ liquid interface. However, the adsorbed films of β -casein at the liquid/liquid interface are more condensed than at the air/liquid interface because interaction with the apolar medium allows extensive loop formation. Hence different structures occur at the two different interfaces (Graham & Phillips, 1980). In the case of globular proteins it is clear from the adsorption isotherm that they

do not adsorb even at low surface coverage in an all train configuration. Some residual elements of the native structure remain. The degree of molecular unfolding at the interface also depends on the packing of the adsorbed material.

The surface rheological parameters reflect the nature of intermolecular interactions in the adsorbed layer. These in turn depend on the degree of unfolding of the molecules. Measurements of dynamic surface tension were therefore made so that information on the relaxation mechanisms could be obtained. The stress relaxation method was used since it is simpler experimentally and a single experiment provides information at all frequencies.

The surface dilatational modulus was measured as a function of frequency bulk concentration and pH at both air/liquid and liquid/liquid interfaces. The initial adsorption is so fast that it is not recorded by this technique. Two further relaxation mechanisms were detected and the characteristic times, τ_1 and τ_2 determined. τ_1 and τ_2 both varied with the above parameters. Cole-Cole plots (Fig.5.12) confirmed the existence of two relaxation processes.

The storage modulus or elasticity, ϵ' (real) rises with frequency reaching a limiting value, ϵ_0 , when there is no exchange between surface and bulk.

τ_1 is a strong function of the surface concentration and decreases as Γ increases at the air/liquid interface. This is due to the fact that as the number of molecules present in the surface increases, the space available for newly adsorbed molecules to unfold decreases and hence the degree of unfolding is less and takes less time.

Because of the interactions of hydrophobic side groups with oil and the possibility of loop formation which penetrate the oil phase,

the variation of τ_1 and Γ is different at the oil/liquid interface. τ_1 , which is associated with the unfolding, is lower at the oil/water interface than at the air/liquid interface as a result of this interaction. With increase in Γ , the value of τ_1 rises. Because the molecules are easier to unfold the time involved in the process is less. As Γ increases the adsorbing molecule takes a longer time to unfold as it must push aside the already adsorbed molecules. This process is possible at the oil/water interface as oil assists in the formation of loops.

The pH of the solution influences the conformation of the dissolved molecules. At pH11 the protein is in its most extended form although it may still have rigid portions when compared with a random coil. When the molecule adsorbs very little time is required for further unfolding. At pH4.7, however, the surface concentration is higher because of the compact conformation of the molecules and this in turn requires a longer time to unfold. At pH7.4 the state is intermediate and the time required is between those corresponding to pH4.7 and pH11.

The results for the oil/water interface show that the greatest time required for unfolding occurs at pH 7.4. At this value of the pH the interfacial tension is a maximum (Fig. 3.12). This implies that the molecule is in its most hydrophilic state and time is required for the hydrophobic moieties to become exposed before they orient and move into the oil phase. At pH11 the interfacial tension is lower than at pH7.4. This is due to the additional flexibility of the chains. The smaller area per molecule which results from loop formation implies that more molecules can adsorb. The time necessary

for the relaxation is lower because the molecule is already in the unfolded state. At pH4.7 the area occupied by a molecule is less than that at pH11. It is therefore concluded that the degree of unfolding is also less and requires a shorter time.

The longer relaxation time, τ_2 , is associated with rearrangements of the molecules within the adsorbed film. After initial unfolding the molecules are not arranged in their most favourable conformational state. A slow readjustment to release strain in the film occurs. At the air/water interface the values of τ_2 decrease with increase in surface concentration.

6.1.3.1 Comparison of τ_1 (fast relaxation process) at the air/water and the oil/water interface

The relaxation times are faster by a factor of about 10 at the oil/water interface than at the air/water interface. This is true for all values of pH. This phenomenon may be explained by the fact that the presence of the oil phase greatly assists in the unfolding process. At the air/water interface the unfolding is probably confined to the initial separation of the domains away from each other. At pH4.7 the molecule is most insoluble exerting maximum hydrophobicity. At other values of pH the molecule in solution is less compact and allows some solvent penetration while at pH11 it has already a fairly open structure as shown by size determination. Hence at pH11 less time is required for unfolding on the surface since the molecule is adsorbed in almost this condition while at pH4.7 where the molecule is most tightly bound the unfolding takes the longest time. At high Γ at the air/water interface, many molecules may not

have space to unfold completely hence the value of τ_1 decreases with increase in Γ . At the oil/water interface, however, since the area available is higher for a given bulk concentration the molecules do unfold even at the highest concentrations although the late arrivals require more time to do so. Hence τ increases with increase in Γ . It is still, however, very much smaller than the values at the air/water interface.

6.1.3.2 Comparison of τ_2 (slow relaxation process) at the air/water and oil/water interfaces

The relaxation times τ_2 are longer at the air/water interface by a factor of 2 than at the oil/water interface. The relaxation is associated with the adsorbed film adjusting itself until it attains a stress free condition. At the air/water interface this involves the movement of whole domains which is a slow process and occurs to a greater extent at low concentrations where there is enough space to make it possible. At high Γ the movement of domains is hindered by the presence of other domains and native molecules. The final state of the film is probably more strained than that at the oil/water interface but the domains form a relatively compact structure.

Again the molecules at the oil/water interface are more expanded and the presence of the oil phase also assists in the relaxation of the stresses in the film. However, the probability of entanglement occurring is greater and should increase at high surface concentration. This is consistent with the increase of τ_2 with Γ . The oil plays a dramatic part in assisting the adjustment of the molecules at the interface and ^{is} shown by the pH dependence. The more hydro-

philic the molecule (pH 7.4) the longer τ_2 . At the air/water interface only the hydrophilic domains can be assisted by the water to leave the interface and relax strains. Hence the relaxation is faster when the molecule is in its most hydrophilic state.

6.2 SUGGESTIONS FOR FUTURE WORK

This research has shown that the conformational changes which occur at interfaces are very sensitive to the nature of the phases in contact. The simplest extension to the work would be to change the polarity of the phase in contact with the protein solution and to relate the differences in rheological behaviour and relaxation phenomena to these changes.

Initial experiments were carried out at interfaces of amylacetate and secondary-butylacetate in contact with water. These indicated that the reduction in interfacial tension by adsorption of HSA is dependent on the interface. Time did not permit a full study of these systems. Other useful phases could consist of low molecular weight polymers.

The work could then be extended to other proteins. The most important factor in choosing other model protein systems is to vary the amount of internal bonding. Thus fibrinogen which denatures to a single thread at the interface would be expected to show marked differences in these parameters. The choice of this and other blood proteins would provide additional information which could be of value in the development of tissue compatible materials.

The expanding drop method for surface rheological measurements is more sensitive to the fast relaxation processes than the trough

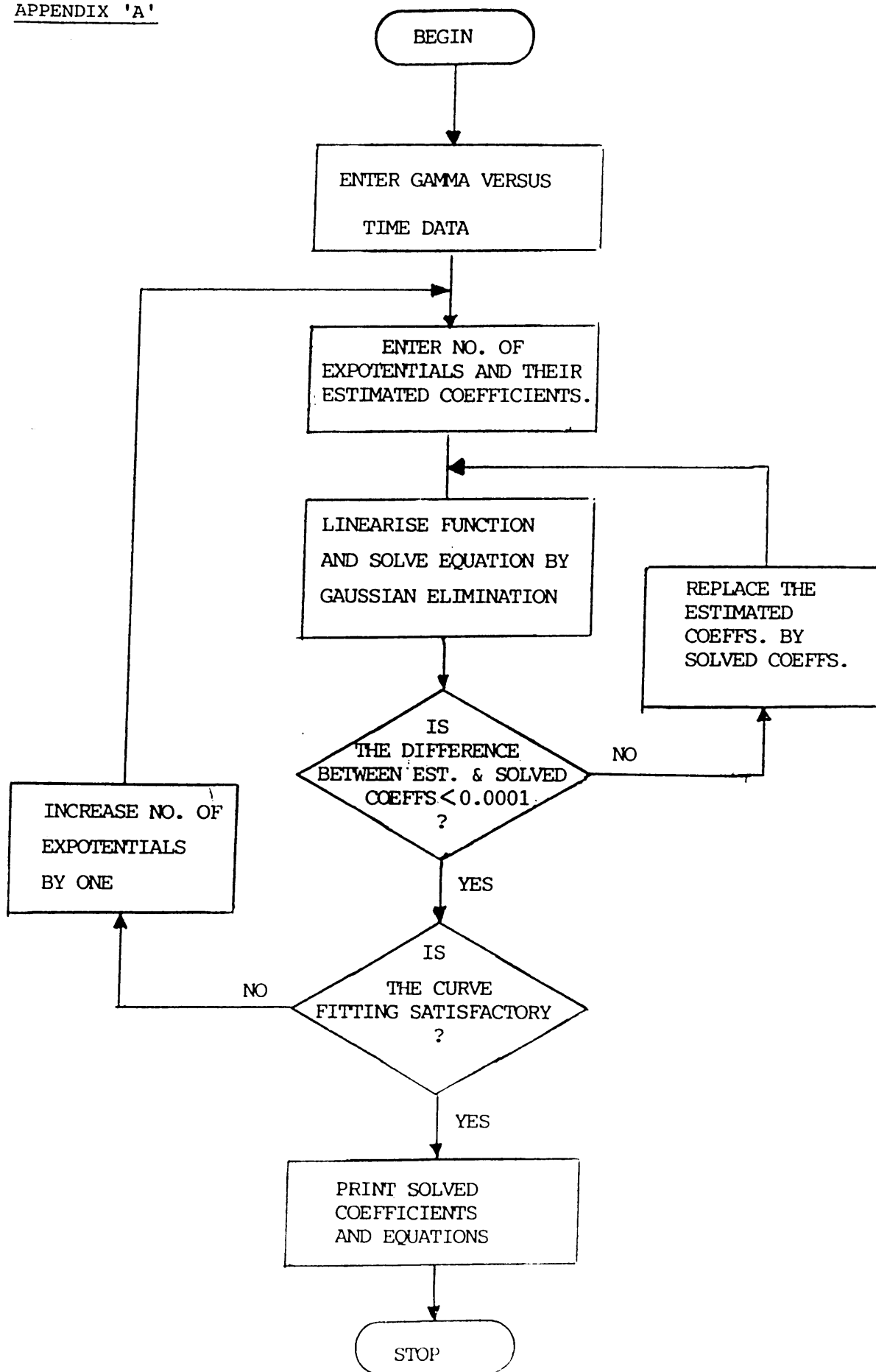
method. It is possible that, with improvement, the equipment might give unambiguous results for processes with characteristic times of less than a second. This implies filtering out all electrical interference and increasing the sensitivity of the pressure transducer. Vibration isolation would also be required.

For studying the initial adsorption other techniques such as light scattering must be used.

Another feature of the system which should be investigated is the distribution of material into the two bulk phases. Some authors have suggested that under some circumstances, e.g. very high bulk concentrations, multilayer adsorption can occur in the aqueous phase near the interface. A radiotracer technique using markers of different intensities could be used in such a study.

Another interesting project would be to study the transfer of materials across the interface, such as cytochrome C and certain enzymes which are known to transfer into iso-octane without loss of activity. The information is useful in the development of techniques used to separate and purify proteins.

Proteins adsorbed at liquid surfaces pose a number of industrial problems. They are instrumental in causing very stable foams in fermenters and in effluent. The surface rheological behaviour which is important in determining foam stability and the effects of additives to improve or hinder foam formation, could be investigated.



PROGRAM LISTING : CURVE FITTING

```

10 PRINT
20 HIMEM: 38399: LOMEM: 24576
30 INPUT "TOTAL NO. OF EXP. EQU. " : M
40 M = M - 1
50 D$ = CHR$(4)
60 DIM A(10), B(10), AI(10), BI(10)
70 DIM C(20), SX(40), SOX(40)
80 DIM G(100), GI(100), T(100)
90 DIM X(20, 20), XO(20, 20)
100 INPUT "DO YOU WISH INPUT DATA FROM DISK OR KEYBOARD (D:K) " : N$
110 IF N$ < > "D" AND N$ < > "K" THEN PRINT CHR$(7) : GOTO 100
120 IF N$ = "D" THEN GOTO 190
130 INPUT "ENTER TOTAL NO. OF DATA " : N
140 PRINT "NOW ENTER ALL DATA"
150 FOR I = 0 TO N - 1
160 PRINT "G(:" I : "): T(:" I : "): " : INPUT G(I), T(I)
170 NEXT I
180 GOTO 270
190 INPUT "ENTER DATA FILE NAME " : NA$
200 PRINT D$ : "OPEN" : NA$
210 PRINT D$ : "READ" : NA$
220 INPUT N
230 FOR I = 0 TO N - 1
240 INPUT T(I) : INPUT G(I)
250 NEXT I
260 PRINT D$ : "CLOSE" : NA$
270 PRINT
280 PRINT "LISTING ALL THE DATA"
290 FOR I = 0 TO N - 1
300 PRINT T(I), G(I)
310 FOR J = 0 TO 200 : NEXT J
320 NEXT I
330 INPUT "DO YOU WISH TO ALTER ANY DATA " : N$
340 IF N$ < > "Y" AND N$ < > "N" THEN PRINT CHR$(7) : GOTO 330
350 IF N$ = "N" THEN GOTO 720
360 PRINT "TO DELETE A DATA TYPE 0000" : PRINT "TO CHANGE A DATA TYPE IN THE CC
RESPONDING CORRECT DATA"
370 FOR I = 0 TO N - 1
380 K = K + 1
390 A$ = ""
400 PRINT "T(:" I : "): G(:" I : "): (" : T(I) : "): (" : G(I) : "): " :
410 GET C$
420 A = ASC(C$)
430 PRINT CHR$(A) :
440 IF A = 13 THEN GOTO 580
450 IF A < 48 OR A > 58 THEN GOTO 480
460 A$ = A$ + C$
470 GOTO 410
480 IF A = 69 OR A = 43 OR A = 45 OR A = 46 THEN GOTO 460
490 IF A = 8 THEN PRINT CHR$(32) : PRINT CHR$(A) : GOTO 540
500 IF A = 32 THEN GOTO 460
510 IF A = 21 THEN GOTO 410
520 IF A = 44 THEN GOTO 460
530 PRINT CHR$(7) : GOTO 460
540 AL = LEN(A$)
550 IF AL < 2 THEN A$ = "" : GOTO 410
560 A$ = LEFT$(A$, AL - 1)

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570 GOTO 410
580 IF A$ = "" THEN GOTO 680
590 IF A$ = "0000" THEN K = K - 1: GOTO 690
600 AL = LEN (A$)
610 FOR J = 1 TO AL
620 SR$ = MID$ (A$,J,1)
630 IF SR$ = "," THEN GOTO 650
640 NEXT J
650 T(K - 1) = VAL ( LEFT$ (A$,J - 1))
660 G(K - 1) = VAL ( MID$ (A$,J + 1))
670 GOTO 690
680 T(K - 1) = T(I):G(K - 1) = G(I)
690 NEXT I
700 N = K:K = 0
710 GOTO 280
720 IF NA$ ( ) "" THEN GOTO 880
730 PRINT "DO YOU WISH TO SAVE THESE DATA ";; INPUT N$
740 IF N$ ( ) "Y" AND N$ ( ) "N" THEN PRINT CHR$ (7): GOTO 730
750 IF N$ = "N" THEN GOTO 880
760 INPUT "ENTER DATA FILE NAME "INA$
770 PRINT D$;"OPEN"INA$
780 PRINT D$;"DELETE"INA$
790 PRINT D$;"OPEN"INA$
800 PRINT D$;"WRITE"INA$
810 PRINT N
820 FOR I = 0 TO N - 1
830 PRINT T(I)
840 PRINT G(I)
850 NEXT I
860 PRINT D$;"CLOSE"INA$
870 PRINT
880 PRINT :XI = 215 / T(N - 1):YY = 115 / G(0)
890 HGR2 : HCOLOR= 3
900 HPLOT 5,5: HPLOT TO 5,120: HPLOT TO 220,120
910 YI = 120
920 FOR I = 0 TO N - 1
930 X = (XI * T(I)) + 5:YX = YY * G(I):Y = YI - YX
940 HPLOT X,Y
950 HPLOT X - 1,Y - 1: HPLOT X + 1,Y + 1: HPLOT X - 1,Y + 1: HPLOT X + 1,Y - 1
960 NEXT I
970 K = K + 1
980 IF K > 1000 THEN GOTO 1020
990 IF PEEK ( - 16384) ( 129 THEN GOTO 970
1000 GET C$
1010 IF ASC (C$) ( ) 13 THEN GOTO 970
1020 K = 0
1030 POKE - 16303,0: POKE - 16300,0
1040 PRINT "ENTER FIRST GUESS OF THE UNKNOWNNS"
1050 FOR J = 0 TO M
1060 PRINT "AI("I;J;"),BI("I;J;") ";; INPUT AI(J),BI(J)
1070 PRINT AI(J),BI(J)
1080 NEXT J
1090 FOR I = 0 TO N - 1
1100 FOR J = 0 TO M
1110 GI = GI + ((AI(J)) * ( EXP ((BI(J)) * T(I))))
1120 NEXT J
1130 GI(I) = GI
1140 PRINT GI;" "IG(I); " "IG(I) - GI
1150 GI = 0
1160 NEXT I

```

```

1170 PRINT
1180 FOR L = 0 TO M
1190 FOR K = 0 TO M
1200 X = 0:Y = 0:Z = 0
1210 P = 0:Q = 0:R = 0:S = 0
1220 FOR I = 0 TO N - 1
1230 T = DT * I
1240 U = EXP ((BI(K)) * T(I))
1250 V = EXP ((BI(L)) * T(I))
1260 X = X + (U * V)
1270 Y = Y + (U * V * T(I))
1280 Z = Z + (U * V * T(I) * T(I))
1290 IF L > 0 THEN GOTO 1320
1300 P = P + ((G(I) - GI(I)) * U)
1310 Q = Q + ((G(I) - GI(I)) * U * T(I))
1320 NEXT I
1330 X((2 * L),K) = X
1340 X((2 * L),(M + K + 1)) = AI(K) * Y
1350 X(((2 * L) + 1),K) = AI(L) * Y
1360 X(((2 * L) + 1),(M + K + 1)) = AI(K) * AI(L) * Z
1370 X = 0:Y = 0:Z = 0
1380 IF L > 0 THEN GOTO 1430
1390 SX(2 * K) = P
1400 SX((2 * K) + 1) = Q * AI(K)
1410 P = 0:Q = 0
1420 R = 0:S = 0
1430 NEXT K
1440 NEXT L
1450 FOR J = 0 TO (2 * M) + 1
1460 FOR K = 0 TO (2 * M) + 1
1470 PRINT X(J,K)
1480 NEXT K
1490 PRINT SX(J)
1500 PRINT
1510 NEXT J
1520 MD = (2 * M) + 1
1530 FOR J = 0 TO MD
1540 FOR K = 0 TO MD
1550 XD(J,K) = X(J,K)
1560 NEXT K
1570 SOX(J) = SX(J)
1580 NEXT J
1590 FOR L = 0 TO MD - 1
1600 FOR I = 0 TO MD
1610 IF X(L,L) = 0 THEN GOSUB 2860
1620 X = X(L,L)
1630 IF X = 0 THEN GOTO 1690
1640 Y = X((I + 1),L)
1650 Z = Y / X
1660 FOR K = L + 1 TO MD
1670 XD = X(L,K) * Z
1680 RX((I + 1),K) = X((I + 1),K) - XD
1690 NEXT K
1700 SDX = SX(L) * Z
1710 RSX(I + 1) = SX(I + 1) - SDX

```

```

1720 NEXT I
1730 FOR J = L + 1 TO MD
1740 FOR K = L + 1 TO MD
1750 PRINT RX(J,K)
1760 NEXT K
1770 PRINT RSX(J)
1780 NEXT J
1790 GOTO 1820
1800 GET C*: IF ASC (C*) = 13 THEN GOTO 1820
1810 GOTO 1800
1820 FOR J = L + 1 TO MD
1830 FOR K = L + 1 TO MD
1840 X(J,K) = RX(J,K)
1850 NEXT K
1860 SX(J) = RSX(J)
1870 NEXT J
1880 PRINT
1890 NEXT L
1900 PRINT
1910 IF X(MD,MD) = 0 THEN C(MD) = 0: GOTO 1930
1920 C(MD) = SX(MD) / X(MD,MD)
1930 PRINT "C("MD:") = "IC(MD)
1940 FOR L = MD - 1 TO 0 STEP - 1
1950 J = L
1960 FOR K = MD TO L + 1 STEP - 1
1970 S = S + C(K) * X(J,K)
1980 NEXT K
1990 IF X(J,K) = 0 THEN C(K) = 0: GOTO 2010
2000 C(K) = (SX(J) - S) / X(J,K)
2010 PRINT "C("IK:") = "IC(K)
2020 S = 0
2030 NEXT L
2040 GOSUB 3000
2050 FOR K = 0 TO M
2060 IF LK > 0 THEN GOTO 2130
2070 A(K) = C(K) + AI(K)
2080 B(K) = C(K + M + 1) + BI(K)
2090 PRINT A(K),B(K)
2100 GOTO 2120
2110 IF A(K) < 0 OR A(K) > 2 * G(0) THEN LK = LK + 1: GOSUB 3140
2120 IF B(K) > 0 THEN LK = LK + 1: GOSUB 3140
2130 NEXT K
2140 PRINT
2150 IF LK > 0 THEN LK = 0: GOTO 1090
2160 PRINT
2170 FOR I = 0 TO N - 1
2180 FOR K = 0 TO M
2190 G = G + (A(K) * EXP (B(K) * T(I)))
2200 NEXT K
2210 PRINT G
2220 G = 0
2230 NEXT I
2240 PRINT
2250 FOR K = 0 TO M
2260 AI(K) = A(K)

```

```

2270 BI(K) = B(K)
2280 NEXT K
2290 J = 0
2300 IF ABS (C(J)) > 1E - 2 OR ABS (C(J + M + 1)) > 1E - 3 THEN GOTO 2330
2310 IF J > M THEN GOTO 2370
2320 J = J + 1: GOTO 2300
2330 GOTO 1090
2340 GET F*: IF ASC (F*) = 13 THEN J = 0: GOTO 1090
2350 IF ASC (F*) = 3 THEN END
2360 GOTO 2340
2370 J = 0
2380 POKE - 16384, 0: POKE - 16288, 0
2390 FOR I = 0 TO N - 1
2400 T = I * DT
2410 FOR J = 0 TO M
2420 IF B(J) > 0 THEN QQ = 1
2430 GI = GI + (A(J) * EXP (B(J) * T(I)))
2440 NEXT J
2450 X = (X1 * T(I)) + 5: YX = YY * GI: Y = Y1 - YX
2460 HPLOT X, Y
2470 GI(I) = GI
2480 GI = 0
2490 NEXT I
2500 K = K + 1
2510 IF K > 1000 THEN GOTO 2550
2520 IF PEEK ( - 16384) < 129 THEN GOTO 2500
2530 GET C*
2540 IF ASC (C*) < 13 THEN GOTO 2500
2550 K = 0
2560 IF QQ = 1 THEN LK = 1: GOSUB 3140
2570 POKE - 16383, 0: POKE - 16300, 0
2580 IF QQ = 1 THEN QQ = 0: GOTO 1090
2590 E = 0: F = 0
2600 EE = 0: FF = 0: EF = 0
2610 FOR I = 0 TO N - 1
2620 DG = ABS (G(I) - GI(I))
2630 E = E + G(I)
2640 F = F + GI(I)
2650 EF = EF + (G(I) * GI(I))
2660 EE = EE + (G(I) + 2)
2670 FF = FF + (GI(I) + 2)
2680 NEXT I
2690 RG = (((E * F) / N) - EF) / (((E + 2) / N) - EE)
2700 AE = E / N
2710 AF = F / N
2720 IN = (AF - (RG * EF))
2730 VE = ((EE / N) - (AE + 2))
2740 VF = ((FF / N) - (AF + 2))
2750 CC = ((RG * VE) / VF)
2760 PRINT "VARIANCE OF ACTUAL VALUES IS : "; VE
2770 PRINT "VARIANCE OF FITTED VALUES IS : "; VF
2780 PRINT "CORRELATION COEFFICIENT IS : "; CC
2790 IF CC > .995 AND RG > .995 THEN GOTO 2850
2800 AO(0) = A(0): BO(0) = B(0)

```

```

2810 GOSUB 3140
2820 PRINT
2830 GOTO 1090
2840 M = M + 1: GOTO 1090
2850 GOTO 3510
2860 W = W + 1
2870 IF (L + W) > MD THEN GOTO 2990
2880 IF X((L + W), L) = 0 THEN GOTO 2860
2890 FOR J = L TO MD
2900 CX = X(L, J)
2910 CW = X((L + W), J)
2920 X(L, J) = CW
2930 X((L + W), J) = CX
2940 NEXT J
2950 S1 = SX(L)
2960 S2 = SX(L + W)
2970 SX(L) = S2
2980 SX(L + W) = S1
2990 RETURN
3000 IN$ = ""
3010 FOR J = 0 TO MD
3020 S = 0
3030 FOR K = 0 TO MD
3040 S = S + (C(K) * XO(J, K))
3050 NEXT K
3060 IF SOX(J) = 0 THEN GOTO 3090
3070 SD = (S / SOX(J)): PRINT "SD" = "SD
3080 IF ABS (SD - 1) > 1E - 2 THEN IN$ = "NOT CORRECT"
3090 PRINT S, SOX(J)
3100 NEXT J
3110 PRINT IN$
3120 IF IN$ ( ) "" THEN END
3130 RETURN
3140 P1 = LOG (G(N - 1))
3150 PRINT "DOING SUB 3000"
3160 XT = 2
3170 IF LK > 0 THEN M = M - 1
3180 P2 = LOG (G(N - XT))
3190 BI(M + 1) = (P1 - P2) / (T(N - 1) - T(N - XT))
3200 AM = (P1 - (BI(M + 1) * T(N - 1)))
3210 AI(M + 1) = EXP (AM)
3220 IF M > 0 THEN GOTO 3340
3230 AI(0) = G(0) - AI(1)
3240 IF AI(0) < 0 THEN XT = XT + 1: GOTO 3180
3250 TR = TR + 2: IF TR > (N - 1) THEN STOP
3260 IF TR = 0 THEN M = M + 1: MM = MM + 2: BI(0) = MM * BI(1): GOTO 3310
3270 PH = (AI(1) * EXP (BI(1) * T(TR)))
3280 BI(0) = (LOG (PH) - LOG (AI(0))) / T(TR)
3290 IF BI(0) > 0 THEN TR = TR - 1: GOTO 3260
3300 M = M + 1
3310 PRINT AI(M - 1), BI(M - 1)
3320 PRINT AI(M), BI(M)
3330 RETURN
3340 AI(0) = AO(0): BI(0) = BO(0)
3350 AW = AW + .2
3360 IF AW = 1 THEN AW = .2
3370 IF AW > .2 THEN GOTO 3390
3380 BW = BW + .05
3390 IF BW = 1 THEN BW = 1.05

```

```

3400 F1 = (.5 * AI(M + 1)) * (1 - AW)
3410 F2 = (.5 * AI(0)) * AW
3420 F3 = (BI(0) - BI(M + 1)) * BW
3430 AI(M) = F1 + F2
3440 BI(M) = (BI(M + 1) + F3)
3450 AI(0) = AI(0) - F2
3460 AI(M + 1) = AI(M + 1) - F1
3470 AI(M) = G(0) - (AI(0) + AI(2))
3480 PRINT AI(0),BI(0); PRINT AI(1),BI(1); PRINT AI(2),BI(2)
3490 M = M + 1
3500 GOTO 3330
3510 PRINT : PRINT D$;"PR#5": PRINT
3520 PRINT TAB( 5)"TIME"; TAB( 6)"ACTUAL DATA"; TAB( 9)"FITTED DATA"; TAB( 9)"
    ACTUAL-FITTED DATA"
3530 PRINT
3540 FOR I = 0 TO N - 1
3550 T$ = STR$(T(I)); G$ = STR$(G(I))
3560 GI$ = STR$(GI(I))
3570 DG = G(I) - GI(I)
3580 DG$ = STR$(DG)
3590 LT = LEN(T$); LG = LEN(G$)
3600 LI = LEN(GI$)
3610 PRINT TAB( 5)T(I); TAB( 10 - LT)G(I);
3620 PRINT TAB( 20 - LG)GI(I); TAB( 20 - LI)DG
3630 NEXT I
3640 PRINT : PRINT : PRINT
3650 FOR J = 0 TO M
3660 A$ = STR$(A(J)); B$ = STR$(B(J))
3670 LA = LEN(A$); LB = LEN(B$)
3680 PRINT TAB( 5)"A(";J;" ) = ";A(J); TAB( 20 - LA)"B(";J;" ) = ";B(J)
3690 NEXT J
3700 PRINT : PRINT D$;"PR#0": PRINT
3710 END

```

```
1490 AB = AB + 1
1500 IF AB > 3 THEN GOTO 1530
1510 PRINT TAB( 17 - BS(I - 1))TL(I);
1520 GOTO 1550
1530 PRINT :AB = 0
1540 PRINT TAB( 12)TL(I);
1550 NEXT I
1560 PRINT
1570 AB = 0
1575 PRINT : PRINT D$:"PR#0": PRINT
1580 PRINT "DO YOU WISH TO SAVE THESE RESULTS": INPUT N$
1590 IF N$ ( ) "Y" AND N$ ( ) "N" THEN PRINT CHR$ (7): GOTO 1580
1600 IF N$ = "N" THEN GOTO 1820
1690 TX = 0
1700 TX = TX + 1
1710 IF TX > 2 THEN GOTO 1820
1720 PRINT D$:"OPEN":A$:"/RESULT":TX
1730 PRINT D$:"DELETE":A$:"/RESULT":TX
1740 PRINT D$:"OPEN":A$:"/RESULT":TX
1750 PRINT D$:"WRITE":A$:"/RESULT":TX
1760 PRINT (LT + 3) / 2
1770 FOR I = 1 TO LT STEP 4
1780 PRINT TL(I): PRINT TL(I + TX)
1790 NEXT I
1800 PRINT D$:"CLOSE":A$:"/RESULT":TX
1810 GOTO 1700
1820 END
```

APPENDIX 'B'

PROGRAM LISTING : FOURIER TRANSFORMATION

```

1  INPUT "TITLE "IA$
5  INPUT "FA "IFA
10 D$ = CHR$ (4)
20 DIM A(3),B(3),F(60),W(60),TL(250)
25 DIM BS(250),FI(10)
26 FI(1) = 1:FI(2) = .8:FI(3) = .6
27 FI(4) = .4:FI(5) = .3:FI(6) = .2
28 FI(7) = .175:FI(8) = .15:FI(9) = .125
29 INPUT "ENTER TOTAL NO. OF EXP. "IEX
30 FOR I = 0 TO EX - 4
40 PRINT "A("II")",B("II") "I: INPUT A(I),B(I)
50 NEXT I
55 LT = 1
60 J = 0
65 DC = 1
67 J = J + 1
70 FC = FC + 1: IF FC = 0 THEN FC = 9:DC = .1 * DC
75 IF FC = 10 THEN FC = 1:DC = 10 * DC
80 F(J) = FI(FC) / DC
90 W(J) = 2 * 3.142 * F(J)
120 FOR I = 0 TO 2
122 S = (A(I) * (W(J) + 2))
124 T = (FA * ((B(I) + 2) + (W(J) + 2)))
130 ER = ER + (S / T)
135 V = (A(I) * B(I) * W(J))
140 EI = EI + (V / T)
150 NEXT I
155 VE = EI / W(J)
156 IF LT > 1 THEN GOTO 160
157 IF EI > .1 THEN FC = FC - 2:J = 0: GOTO 160
158 IF EI < .05 THEN 160
160 TL(LT) = F(J):TL(LT + 1) = ER:TL(LT + 2) = EI
165 TL(LT + 3) = VE
167 IF EI < .1 AND ER < .01 THEN GOTO 195
170 LT = LT + 4
175 PRINT F(J): "IER:" "IEI
180 ER = 0:EI = 0
190 GOTO 67
195 PRINT
200 PRINT D$:"PR#5"
1400 PRINT : PRINT
1410 PRINT TAB( 12)"FREQ          E(REAL)          E(IMAG)          VISC"
1420 PRINT TAB( 12)"-----          -----          -----          ----"
1430 PRINT TAB( 12)"(HZ)          (MN/M)          (MN/M)          (MNS/M)"
1440 PRINT
1450 PRINT TAB( 12)TL(1):
1460 BC$ = STR$ (TL(1)):BS(1) = LEN (BC$)
1470 FOR I = 2 TO LT + 3
1480 BC$ = STR$ (TL(I)):BS(I) = LEN (BC$)

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REFERENCES

- Absolom, D.R., Newmann, A.W. & van Oss, C.J. Am. Chem. Soc. Symposium 240, 169 (1984).
- Adamson, A.W. Physical Chemistry of Surfaces. Wiley - Interscience Pub., New York (1976).
- Alexander, P. & Hamilton, L.D.G. Arch. Biochem. Biophys. 88, 128 (1960).
- Allen, P.C., Hill, E.A. & Stokes, A.M. 'Plasma Proteins', London (1977).
- Andrade, J.D. Medical instrumentation 7, 110 (1973).
- Anderson, L.O. Biochim. Biophys. Acta 117, 155 (1966).
- Anderson, S.R. & Weber, G. Biochemistry 8, 371 (1969).
- Andreas, J.M., Hauser, E.A. & Tucker, W.B. J. Phys. Chem. 12, 1001 (1938).
- Augenstein, L.G. & Ray, B.R. J. Phys. Chem. 61, 1380 (1957).
- Augenstein, L.G., Ghiron, C.A. & Nims, L.F. J. Phys. Chem. 62, 1231 (1958).
- Aniansson, G. J. Phys. Chem. 55, 1286 (1951).
- Bagnall, R. Chem. in Britain 14, No. 12, p.598 (1978).
- Baier, R.E. & Zisman, W.A. Rep. N. NRL 6755, Nav. Res. Lab. Washington, D.C. (1968).
- Baier, R.E. & Dulton, R.C. J. Biomed. Mater. Res. 3, 191 (1969).
- Baier, R.E. & Zisman, W.A. Macromolecules, 3, 70 (1970a).
- Baier, R.E. & Zisman, W.A. Macromolecules, 3, 462 (1970b).
- Bamford, C.H., Elliot, A. & Hanby, W.E. Synthetic Polypeptides, p.355, Academic Press New York (1956).
- Bashforth, F. & Adams, J.C. An attempt to test the theories of capillary action. University Press, Cambridge, England (1883).
- Behrens, P.Q., Spiekerman, A.M. & Brown, J.R. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 34, 591 (1975).

- Bendure, R.L. J. Coll. & Int. Sci. 35, 238 (1971).
- Benhamon, N. & Guastalla, J. J. Chim. Phys. 57, 745-751 (1960).
- Benjamins, J., de Feitjar, J.A., Evans, M.T.A., Grahams, D.E. & Phillips, M.C. Dis. Farad. Soc. 59, 218 (1925).
- Bigelow, G.C. J. Theor. Biol. 16, 187 (1967).
- Blank, M., Luccasen, J. & Van den Tempel, M. J. Coll. & Int. Sci. 33, 94 (1970).
- Bloomfield, V. Biochemistry 5, 684 (1966).
- Bornzin, A.G. & Miller, I.F. J. Coll. & Int. Sci., 86, 539 (1982).
- Brash, J.L., Lyman, D.J. J. Biomed. Mater. Res. 3, 175 (1969).
- Brash, L.J. & Samak, Q.M. J. Coll. & Int. Sci. 65, 495-504 (1978).
- Brier-Russel, D., Salzman, E.W., Handin, R., Merrill, E.W., Dincer, A.K. & Wu, S.K. J. Coll. & Int. Sci. 81, 311 (1981).
- Brown, J.R., Low, T., Behrens, P., Sepulveda, P., Parker, K. & Blackney, E. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 30, 1104 (1971).
- Brown, J.R. Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33, 1389 (1974).
- Brown, J.R. Fed. Proc., Fed. Amer. Soc. Exp. Biol. 34, 591 (1975).
- Bull, H.B. Advan. Prot. Chem. 3, 95 (1947).
- Bull, H.B. Biochem. Biophys. 59, 53 (1955).
- Bull, H.B. Biochem. Biophys. Acta 19, 464 (1956)
- Bull, H.B. Arch. Biochem. Biophys. 68, 102 (1957).
- Bull, H.B. Arch. Biochem. Biophys. 98, 427 (1962).
- Callaghan, I.C., Gould, C.M., Hamilton, R.J. & Neustadter, E.L. Coll. & Surf. 8, 17-28 (1983).
- Camejo, G., Colacicco, G. & Rapport, M.M. J. Lipid Res. 9, 562 (1968).
- Champagne, M. J. Polym. Sci. 23, 863 (1957).
- Cheesman, D.F. & Schuller, H. J. Coll. Sci. 9, 113 (1954).

- Clauser, H. & Li, C.H. J. Am. Chem. Soc. 76, 4337 (1954).
- Cohn, E.H., Strong, L.E., Hughes, W.L.Jr., Mulford, D.J., Ashwother, J.N.,
Melim, M. & Taylor, H.L. J. Amer. Chem. Soc. 68, 459 (1946).
- Coleman, D.L., Atwood, A.I. & Andrade, J.D. J. Bioeng. 1, 33 (1976).
- Cosgrove, T., Vincent, B., Crowley, T.L. & Cohen Stuart, M.A. in Polymer
Adsorption and Dispersion Stability. A.C.S. Symp. Ser. 240, 147
(1984)
- Cumper, C.W.N. & Alexander, A.E. Trans. Farad. Soc. 46, 235-243 (1950).
- Cumper, C.W.N. & Alexander, A.E. Trans. Farad. Soc. 46, 243-252 (1950).
- Davies, J.T. & Rideal, E.K. Interfacial Phenomena. Academic Press (1963).
- De Feijter, J.A., Benjamins, J. J. Coll. & Int. Sci. 90, 293 (1982).
- De Gennes, P.G. J. Phys. 37, 1445 (1976).
- De Gennes, P.G. 'Scaling concepts in polymer physics', Cornell University
Press (1979).
- De Gennes, P.G. Macromolecules 14, 1637 (1981).
- De Gennes, P.G. Macromolecules 15, 492 (1982).
- De Gennes, P.G. & Pincus, P. J. de Phys. Lettr. 44, 1241 (1983).
- Devaux, H. J. Phys. Radium 699, 891 (1912).
- Devaux, H. J. Physique, Vol. 3, p.450 (1904).
- Dewey, W.C. Amer. J. Physiol. 197, 423 (1959).
- Djabbarah, N.F. & Wasan, D.T. Chem. Eng. Sci. 37, 175 (1982).
- Edward, F.B., Rombauer, R.B. & Campbell, B.J. Biochim., Biophys. Acta.
194, 234 (1969).
- Eugene, A. 'Biophysical Science'. Prentice-Hall International, London
(1962).
- Evans, M.T.A., Mitchell, J., Musselwhite, P.R., Irons, L. In 'Surface
Chemistry' (Blank, M. ed.) 1, Plenum Press, N.Y. (1970).

- Evans, M.T.A., Mitchell, J., Musselwhite, P.R., Irons, L. Advan. Exp. Med. Biol. 7, 1 (1970).
- Eylar, E.H. J. Theor. Biol. 10, 89 (1965).
- Fantl, P. Comp. Biochem. Physiol. B42, 403 (1972).
- Feien, J. Thrombogenesis caused by blood foreign surface interactions. In Artificial Organs, MacMillan, London (1977).
- Fink, D.J. & Grendreau, M.R. Analyt. Biochem. 139, 140 (1984).
- Flory, P.J. J. Chem. Phys. 9, 660 (1941)
- Foster, J.F. "The plasma proteins". F.W. Putnam, Ed. Vol. 1, p.179 Academic Press, N.Y. (1960).
- Fowkes, F.M. Ind. & Eng. Chem. 56, 12 (1964).
- Frisch, H.I. & Simha, R. J. Chem. Phys. 24, 4, 652 (1956).
- Frisch, H.I. & Simha, R. J. Chem. Phys. 27, 3, 702 (1957).
- Fukawa, K., Asakura, T. & Daimon, H. Kobunshi Kagaku 18, 596 (1961).
- Garret, W.D. & Zisman, W.A. J. Phys. Chem. 74, 1796 (1970).
- Ghosh, S. & Bull, H.B. Biochim. Biophys. Acta 66, 150-153 (1963).
- Ghosh, S., Breese, K. & Bull, H.B. J. Coll. Sci. 19, 457 (1964).
- Gibbs, J.W. The Collected Works of J.W. Gibbs. Longmans, Green, New York (1931).
- Glass, J.E. J. Phys. Chem. 72, 4450 (1968).
- Gordon, A.H. & Humphrey, J.H. Biochem. J. 78, 551 (1961).
- Gorman, R.R., Stoner, G.E. & Cat Lin, A. J. Phys. Chem. 75, 14, 2103-2107 (1971).
- Gorter, E. Ann. Rev. Biochem. 10, 619 (1941).
- Graham, D.E. & Phillips, M.C. J. Coll. & Int. Sci. Vol. 70, 415 (1979)
- Guest, P.G. 'Numerical methods of curve fitting'. Cambridge Univ. Press (1961).

- Hansen, R.S. & Ahmed, J. in "Progress in surface and membrane science",
Vol. 4, p.5. Academic Press, N.Y. (1971).
- Haurowitz, F., Boucher, P., Dicks, M. & Therriault, D. Arch. Biochem.
Biophys. 59, 52 (1955).
- Hlady, V. & Furedi-Milhoffer, H. J. Coll. & Int. Sci. 69, 460 (1979).
- Harkins, W.D., Fourt, L. & Fourt, P.C. J. Biol. Chem. 132, 111 (1940).
- Harkins, W.D. Colloid Chemistry, Vol. 5, 35-41 (1944).
- Helfand, E., Frisch, H.L. & Lebowitz, J.L. J. Chem. Phys. 34, 1037
(1961).
- Hoeve, C.A.J. J. Polym. Sci. 34, 1-10 (1971).
- Hoeve, C.A.J. J. Polym. Sci. C, 30, 361 (1970).
- Horan, F.E., Hrisch, F.G., Wood, L.A. & Wright, I.S. J. Clin. Invest. 29,
202 (1950).
- Huggins, M.L. J. Chem. Phys. 9, 440 (1941).
- Huggins, M.L. J. Phys. Chem. 46, 151 (1942).
- Hughes, W.L. 'The Proteins', Academic Press, New York (1954).
- Humphrey, J.H., Neuberger, A. & Perkins, D.J. Biochem. J. 66, 390 (1957).
- Hunter, J.M. & McDuffie, F.C. J. Amer. Chem. Soc. 81, 1400 (1959).
- James, L.K. & Labows, J.N. J. Phys. Chem. 68, 1122 (1964).
- James, L.K. & Augenstein, L.G. Advances in Enzymology 28, 1 (1966).
- Kabat, E.A. & Heidelberger, M. J. Exp. Med. 66, 229 (1937).
- Kato, T., Nakamina, N., Kawaguchi, M. & Takahashi, A. Polym. J. 13, 1037
(1981).
- Katz, J., Bonorris, G. & Sellers, A.L. Clin. Sci. 39, 705 (1970).
- Kauzmann, W. & Simpson, R.B. J. Amer. Chem. Soc. 75, 5154 (1953).
- Keating, F.R. Jr., Jones, J.D., Elveback, L.R. & Randall, R.V. J. Lab.
Clin. Med. 73, 825 (1969).

- Keenan, M. J. Phys. Chem. Vol. 33, p.371 (1929).
- Kelman, L., Saunders, S.J., Frith, L., Wicht, S. & Corrigan, A. Amer. J. Clin. Nutr. 25, 1174 (1972).
- Kelvin, L. (W. Thompson) Phil. Mag. 42, 368 (1871).
- Kim, S.W. Platelet protein interactions. Bioengineering Seminar on Artificial Organs, University of Strathclyde (1976).
- King, T.P. & Spencer, M. Arch. Biochem. Biophys. 153, 627 (1972).
- Kiosseoglou, V.D. & Sherman, P. Coll. & Polym. Sci. 261, 502 (1983).
- Kresheck, G.C. & Klotz, I.M. Biochem. 8, 8 (1969).
- Kusnir, J. & Meloun, B. Collect. Czech. Chem. Commun. 38, 143 (1973a).
- Kusnir, J. & Meloun, B. Biochim. Biophys. Acta 310, 124 (1973b).
- Lamb, Sir H. 'Hydrodynamics', 6th Ed. Dover, New York (1945).
- Lampert, H. Muenchen Med. Wochenschr. 77, 586 (1930).
- Langmuir, I. & Schaefer, V.J. J. Amer. Chem. Soc. 60, 1351 (1938).
- Langmuir, I. & Schaefer, V.J. Chem. Rev. 24, 181 (1939).
- Lankveld, J.M.G. & Lyklema, J. J. Coll. & Int. Sci. 41, 454 (1972).
- Lee, R.G., Adamson, C., Kim, S.W. Thromb. Res. 4, 485 (1974).
- Lee, R.G. & Kim, S.W. J. Biomed. Mater. Res. 8, 251-259 (1974).
- Leininger, R.I., Mirkovitch, V., Beck, R.E., Andreas, P.G. & Kloff, W.J. Trans. Amer. Soc. Artif. Intern. Organs 10, 237 (1964).
- Leininger, R.I., Falb, R.D. & Grode, G.A. Ann. N.Y. Acad. Sci. 148, Art. 1-11 (1968).
- Levine, S. Arch. Biochem. Biophys. 50, 515 (1954).
- Llopis, J. & Rebollo, D. Arch. Biochem. Biophys. 88, 142 (1960).
- Loeb, G.K. & Baier, R.E. J. Coll. & Int. Sci. 27, 38 (1968).
- Loeb, G.I. J. Coll. & Int. Sci. 31, 572 (1969).
- Loglio, G., Tesei, U. & Cini, R. J. Phys. Chem. 81, 1154 (1977).

- Loglio, G., Tesei, U. & Cini, R. J. Coll. & Int. Sci. 71, 316 (1979).
- Lok, B.K., Cheng, Y.L. & Robertson, C.R. J. Coll. & Int. Sci. 91, 87 (1983).
- Lok, B.K., Cheng, Y.L. & Robertson, C.R. J. Coll. & Int. Sci. 91, 104 (1983).
- Longworth, L.G. J. Am. Chem. Soc. 75, 5705 (1953).
- Longworth, L.G. & Jacobsen, C.F. J. Phys. Coll. Chem. 53, 126 (1949).
- Lucassen, J. & Hansen, R.S. J. Coll. & Int. Sci. 23, 319-329 (1967).
- Lucassen, J. Trans. Farad. Soc. 65, 2221 (1968).
- Lucassen-Reynders, E.H. & Lucassen, J. Adv. in Coll. & Int. Sci. 2, 347 (1969).
- Lucassen, J. & Van den Tempel, M. J. Coll. & Int. Sci. 41, 491 (1972).
- Lucassen, J. & Van den Tempel, M. Chem. Eng. Sci. 27, 1283 (1972).
- Lucassen, J. & Barnes, G.T. J. Chem. Soc. Farad. Trans. 1, 68, 2129 (1972).
- Lucassen-Reynders, E.H., Lucassen J., Barret, P.R., Giles, D. & Holloway, F. Advances in Chemistry Series 144, 272 (1975).
- Lucassen, J. & Giles, D. J. Chem. Soc. Farad. Trans. 1, 71, 217 (1975).
- Lyman, D.J. Rev. Macromolecule Chem. 1, 355 (1966).
- Lyman, D.J. Ann. N.Y. Acad. Sci. 148, Art 1, 30, 113 (1968).
- Lyman, D.J. & Kim, S.W. Fed. Proc. Fed. Am. Soc. Exp. Biol. 30, 1658 (1971).
- McBain, J.W. & Swain, R.C. Proc. Royl. Soc. A154, 608 (1936).
- McBain, J.W. & Wood, L.A. Proc. Royl. Soc. A174, 286 (1940a).
- McBain, J.W., Mills, G.F. & Ford, T.F. Trans. Farad. Soc. 36, 931 (1940b).
- MacRitchie, F. & Alexander, A.E. J. Coll. Sci. 16, 57-67 (1961).
- MacRitchie, F. & Alexander, A.E. J. Coll. Sci. 18, 464-469 (1963).

- MacRitchie, F. & Alexander, A.E. *J. Coll. & Int. Sci.* 18, 453, (1963).
- Maru, H.C. & Wasan, D.T. *Chem. Eng. Sci.* 34, 1283 (1979)
- Masat, R.J. & Dessauer, C. *Comp. Biochem. Physiol.* 25, 119 (1968).
- Matthews, B.L. *Trans. Farad. Soc.* 35, 1113 (1939).
- McLaren, A.D. in 'Cell Interface Reaction', H.D. Brown, ed. N.Y. p.1 (1963).
- Meloun, B. & Kusnir, J. *Fed. Eur. Biochem. Soc. Lett.* 27, 121 (1972).
- Metcalf, K. *J. Phys. Chem. Vol.* 52, p.1 (1905).
- Miller, L.L., Bly, C.G., Watson, M.L. & Bale, W.F. *J. Exp. Med.* 94, 431 (1951).
- Minamatsu (1978).
- Morrissey, B.W. & Han, C.C. *J. Coll. & Int. Sci.* 65, 423 (1976).
- Motomura, K. & Matura, R. *J. Coll. Sci.* 18, 52 (1963).
- Muramatsu, M. *Surfaces & Coll. Sci.* 6, 101-184 (1973).
- Nelson, C.E. & Guttman, S.I. *Comp. Biochem. Biophys.* B44, 423 (1973).
- Norde, W. & Lyklema, J. *J. Coll. & Int. Sci.* 71, 2, 350 (1979).
- Otten, J., Janckheer, M. Dumont, J.E. *J. Clin. Endocrinol Metals*, 32, 18 (1971).
- Orveskes, I. & Singer, J.M. *J. Immunol.* 86, 338 (1961).
- Peters, T.Jr. *J. Biol. Chem.* 237, 1181 (1962).
- Peters, T.Jr. *Advan. Clin. Chem.* 13, 37 (1970).
- Phillies, G.D.J., Benedek, G.B. & Mazer, N.A. *J. Chem. Phys.* 65, 1883 (1976).
- Privalov, P.L. & Krechinashvili, J. *J. Mol. Biol.* 86, 665 (1974).
- Putnam, F.W. 'The plasma proteins', Vol. 1, London (1975).
- Putnam, F.W. 'The plasma proteins', Vol. 4, London (1975).
- Rahman, A., Sherman, P. *Colloid & Polymer Sci.* 260, 35 (1982).

- Ray, B.R. & Auugenstein, L.G. J. Phys. Chem. 60, 1193 (1956).
- Rillaerts, E. & Joos, P. J. Coll. & Int. Sci. Vol. 88, 1 (1982).
- Roe, R.J., Bacchetta, V.L. & Wong, P.M.G. J. Phys. Chem. 71, 4190 (1967).
- Roe, R.J. J. Chem. Phys. 60, 4192 (1974).
- Rosenfeld, M. & Surgenor, D.M. J. Biol. Chem. 183, 663 (1950).
- Rose, J.C. & Broida, H.F. Proc. Soc. Exp. Biol. Med. 86, 384 (1954).
- Ross, J., Greenfield, L.J., Bowman, R.L. & Morrow, A.G. Prosthetic valves for cardiac surgery, p.212, Thomas Springfield, Illinois, (1961).
- Rothschild, M.A., Bauman, A., Yalow, R.S. & Berson, S.A. J. Clin. Invest. 34, 1354 (1955).
- Russell, D.B., Salzman, E.W., Handin, R. & Wu, J.S. J. Coll. & Int. Sci. 81, 2, 311 (1981).
- Sa Da Costa, V., Brier-Russel, D., Trudel, G., Wolfe, L., Waugh, D., Salzman, E.W. & Merril, E.W. J. Coll. & Int. Sci. 76, 594 (1980).
- Sawyer, P.N., Burrowes, C., Oganiak, I., Smith, A.D. & Weselowski, S.A. Trans. Amer. Soc. Artif. Intern. Organs 10, 317 (1964).
- Scheutjens, J.M.H.M. & Fleer, G.H. J. Phys. Chem. 83, 1619 (1979).
- Scheutjens, J.M.H.M. & Fleer, G.H. J. Phys. Chem. 84, 178 (1980).
- Scheutjens, J.M.H.M. & Fleer, G.H. Adv. Coll. & Int. Sci. 16, 341 (1982).
- Shearer, W.T., Bradshaw, R.A., Gurd, F.R.N. & Peters, T. Jr. J. Biol. Chem. 242, 5451 (1967).
- Schaaf, P. & Dejardin, Ph. Coll. & Surf. 24, 239 (1987).
- Scheller, F., Janchen, M. & Prumke, H.J. Biopolymers 14, 1553 (1975).
- Silberberg, A. J. Phys. Chem. 66, 1884-1907 (1962).
- Silberberg, A. J. Polym. Sci. C.30, 393-397 (1970).
- Singer, S.J. J. Chem. Phys. 16, 872 (1948).

- Slayter, E.M. J. Mol. Biol. 14, 443 (1965).
- Smith, L.E., Ferstermaker, C.A. & Stromberg, R.R. Polym. Prep. 11, 1376 (1970).
- Sorbo, B. Acta. Chem. Scand. 15, 139 (1961).
- Stauff, J. & Jaenicke, R. Kolloid 2, 175, 1 (1961).
- Steiner, R.F. 'Life Chemistry' Pub. by D. van Nostrand, Co. Ltd., London (1968).
- Steinhardt, J. & Stocker, N. Biochemistry 12, 1789 (1973).
- Stokes, G.G. Trans. Cambridge Phil. Soc. 8, 441 (1847).
- Stroupe, S.D. & Foster, J.F. Biochemistry 12, 3824 (1973).
- Tanford, C. Adnan. Prot. Chem. 17, 69 (1962).
- Tanford, C., Kawahara, K. & Lapanje, S. J. Amer. Chem. Soc. 89, 29 (1967).
- Tanford, C. Advan. Prot. Chem. 24, 1 (1970).
- Ting, L., Wasan, D.T., Miyano, K. & S-Q Xu. J. Coll. & Int. Sci. 102, 248 (1984).
- Traube, Liebigs Ann. 265, 27 (1891).
- Trunnit, H.J. J. Coll. Sci. 15, 1 (1960).
- Ullman, R., Koral, J. & Eirich, F.R. Proc. 2nd Intern. Congr. Surface Activity 3, 485 (1952).
- Van den Linden, C. & Van Leemput, R. J. Coll. Int. Sci., 67, 48 and 63 (1978).
- Van den Tempel, M. & Van de Reit, R.P. J. Chem. Phys. 42, 2769 (1965).
- Van Oss, C.J., Good, R.J. & Chaudhury, M.K. J. Coll. & Int. Sci., 111, 2, 378-390 (1986)
- Van Oss, C.J., Good, R.J. & Chaudhury, M.K. In press.
- Van Voorst Vader, F., Erkens, F.Th., & Van den Tempel, M. Trans. Farad. Soc. 60, 1170 (1964).

- Veer, F.A. & Van den Tempel, M. J. Coll. & Int. Sci. 42, 418 (1973).
- Vroman, L. Nature, London, 196, 476 (1962).
- Vroman, L., Adams, A.L. & Klings, M. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 30, 1494 (1971).
- Vroman, L. & Adams, A.L. Thromb. Diath. Haemorrh. 18, 510 (1967).
- Wallace, D.G. & Wilson, A.C. J. Mol. Evol. 2, 72 (1972).
- Wallerik, K. J. Biol. Chem. 248, 2650 (1973).
- Ward, A.F.H. & Tordai, L. J. Chem. Phys. 14, 453 (1946).
- Weber, G. & Young, L.B. J. Biol. Chem. 239, 1415 (1964).
- Whitney, R.McL. In 'Food Colloids' (H.D. Graham, ed.) 88, AVI Publishing Co., Westport, Conn., U.S.A. (1977).
- William, D.F. & Bagnall, R.D. 'Fundamental Aspects of Biocompatibility' 113 (1981).
- Yamashita, T. & Bull, H.B. J. Coll. & Int. Sci. 24, 310 (1967).
- Yudenfreund, M.N., Becher, P., Brown, J., in 'Monolayers' (E.D. Goddard ed.) p.192, American Chemical Society (1975).
- Zittle, C.A. Advances in Enzymology, Vol. 14, 319 (1953).